Harnessing Resistance-Nodulation-Division Family Transporters to Modify Cellular Secretion in *Synechocystis* sp. PCC 6803

by

Matthew Bellefleur

A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Approved April 2018 by the
Graduate Supervisory Committee:

Roy Curtiss III, Chair
David Nielsen
Bruce Rittmann
Xuan Wang

ARIZONA STATE UNIVERSITY

December 2018

ABSTRACT

Synechocystis sp. PCC 6803 is a readily transformable cyanobacteria used to study cyanobacterial genetics, as well as production of biofuels, polyesters, and other industrial chemicals. Free fatty acids are precursors to biofuels which are used by *Synechocystis* cells as a means of energy storage. By genetically modifying the cyanobacteria to expel these chemicals, costs associated with retrieving the products will be reduced; concurrently, the bacteria will be able to produce the products at a higher concentration. This is achieved by adding genes encoding components of the *Escherichia coli* AcrAB-TolC efflux system, part of the resistance-nodulation-division (RND) transporter family, to *Synechocystis* sp. PCC 6803. AcrAB-TolC is a relatively promiscuous multidrug efflux pump that is noted for expelling a wide range of substrates including dyes, organic solvents, antibiotics, and free fatty acids. Adding components of the AcrAB-TolC multidrug efflux pump to a previously created high free fatty acid producing strain, SD277, allowed cells to move more free fatty acids to the extracellular environment than did the parent strain. Some of these modifications also improved tolerance to antibiotics and a dye, rhodamine 6G. To confirm the function of this exogenous efflux pump, the genes encoding components of the AcrAB-TolC efflux pump were also added to *Synechocystis* sp. PCC 6803 and shown to grow on a greater concentration of various antibiotics and rhodamine 6G. Various endogenous efflux systems have been elucidated, but their usefulness in expelling products currently generated in *Synechocystis* is limited. Most of the elucidated pumps in the cyanobacteria are part of the ATP-binding cassette superfamily. The knowledge of the resistance-nodulation-division (RND) family transporters is limited. Two genes in *Synechocystis* sp. PCC 6803, *slr2131* and *sll0180*

encoding homologs to the genes that encode acrB and acrA, respectively, were removed and the modifications resulted in changes in resistance to various antibiotics and a dye and also had an impact on free fatty acid secretion. Both of these deletions were complemented independently with the homologous *E. coli* gene and the resulting cyanobacteria strains had some of the inherent resistance restored to chloramphenicol and free fatty acid secretion was modified when compared to the wild-type and a high free fatty acid producing strain.

ACKNOWLEDGMENTS

I would like to thank Dr. Roy Curtiss III for allowing me to join his esteemed research family. I had never performed a PCR, having only loaded one well of an electrophoresis gel. Nor had I ever used an autoclave or made media for bacteria, but at least I had used a micropipette. I had never designed and built my own research project and yet with the gentle urging of Dr. Brenda Hogue, my first supporter at Arizona State, Dr. Curtiss accepted me into his laboratory. I was warmly greeted by Soo-Young Wanda, who has spent the last 6 years teaching, advising, and supporting me through my days in the lab, always able to offer advice and laugh at my mistakes. I could not have finished without her. I would also like to thank my committee members, Dr. David Nielsen, Dr. Bruce Rittmann, and Dr. Xuan Wang, whose questions still ring in my head as I am writing this message of gratitude. From Dr. Rittmann's big picture questions about ultimate goals and purposes, to Dr. Nielsen and Dr. Wang's more poignant remarks regarding specific experiments and results, I owe much of the evolution of this dissertation research to them. I am grateful to the many members of the Curtiss research group who have been a consistent support group, helping me through experiments theoretically and physically.

I would also like to thank my best friends that I rowed with for four years at the University of Michigan. I have been there to watch many of them get married and start their professional lives since we graduated college. We have constantly shared the ups and downs of Michigan athletics (mostly ups for the rowing team). They are always there to listen to my grievances, successes, and willing to relax a couple of times per year to recharge from our respective day jobs.

And finally, thank you to my family including my wife, parents, and brother. I met my wife about halfway through the process when I was still trying to figure out the goals of all of this. She has been my motivation throughout the last 3 years and even helped me around the lab as time got tight at the end. She is a talented chef and brilliant scientist with whom I am lucky to spend my life. My parents inspired me to pursue an education in science through their fruitful decades of saving lives, always providing advice and consultation about anything from politics to whether or not I really have time to finish just that one last experiment. My brother has also been a constant positive force in my life as he heads towards a career in counseling drug addicted teenagers. He inspires the best in me in with his altruistic goals of helping young adults through the most trying times in their lives.

TABLE OF CONTENTS

Page

CHAPTER Page

APPENDIX Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

LIST OF ABBREVIATIONS

3HB	3-hydroxybutyrate
ABC	ATP-binding cassette
ACC	Acyl-ACP thioesterases and acetyl-CoA carboxylase
ACP	Acyl-acyl carrier protein
Amp	Ampicillin
Cm	Chloramphenicol
FFA	Free fatty acid
GC	Gas chromatograph
IC_{50}	Half maximal inhibitory concentration
Kan	Kanamycin
LB	Lysogeny Broth
MATE	Multi Antimicrobial extrusion
MFP	Membrane fusion protein
MFS	Major facilitator superfamily
MIC	Minimum inhibitory concentration
OD	Optical Density
PBP2	Penicillin-binding protein 2
PCR	Polymerase Chain Reaction
RND	Resistance-nodulation-division
RT-PCR	Reverse transcriptase-polymerase chain reaction
R6G	Rhodamine 6G
SDS	Sodium dodecyl sulfate

SMR	Small multidrug resistance
Spec	Spectinomcin
Str	Streptomycin
TEM	Transmission Electron Microscopy
TES	N-[tris(hydroxymethyl) methyl]-2-aminoethanesulfonic acid

CHAPTER 1

INTRODUCTION

1.1 Impetus and Technological Context

Cyanobacteria established our oxygenated atmosphere approximately 2.68 billion years ago, based on shales observed in Pilbara Craton, Australia, which are the only two preserved Archean crusts (1). The percent of oxygen in the atmosphere increased in a step-wise fashion; although, there is some disagreement as to what caused each step Each step may correspond to major collision of Earth's landmasses. Every continental collision created supermountains that were unstable and prone to eroding from wind and water. This erosion led to vast releases of large quantities of iron and phosphorus into the ocean in a relatively small amount of time (2). These element depositions into the ocean created similar algal and cyanobacteria blooms to those that are currently observed in the Gulf of Mexico and other regions throughout the world. Alternatively, other scientists have suggested that the steps were a result of changes in the temperature of the atmosphere, more specifically glaciation events where oxygenic cyanobacteria destroyed the methane greenhouse (3). Irrespective of the process of oxygenation, the resulting oxygenated atmosphere allowed for the evolution of a far more energy-intensive organism, dependent on aerobic respiration, which is about 18 times more efficient than anaerobic respiration (4). Through the millennia, large carbon sinks increased, buried in the sediments and swamps of earth as aerobically respiring organisms died. Coal, the fuel that drove much of the industrial revolution and continues to drive many factories and power plants around the world, originated predominantly from plants, algae, and fungi (5). But petroleum, which has a much greater distribution around the world is derived from algae,

cyanobacteria, and ocean plants predominantly as was shown in a 1937 comparison of the composition of the various hydrocarbons in existence in petroleum reserves and the temperatures at which they can form (6).

These consumed fossil fuels that are used with little regard to the consequences, are derived from the same organisms that continue to oxygenate the atmosphere while simultaneously counteracting the effects of increased greenhouse gas emissions. The latest World Meteorological Organization Greenhouse Gas Bulletin explained that 2016 was the warmest year on record at 1.1^{o}C average air temperature above pre-industrial levels (0.06^{o}C above 2015), due, in part, to an increased atmospheric CO_2 concentration (400ppm in 2015 to 403.3 in 2016) (7). This increasing greenhouse gas pollution stems from fossil fuel use; it is also a result of land-use changes from forest to industrial farms for livestock and crops, some of which are used to make cellulosic-based ethanol for fuel (8, 9). Currently, traditional land plants (corn, sawgrass, e.g.) are used to produce ethanol, which can be utilized as an additive to gasoline theoretically in order to reduce greenhouse gas emissions (10). However, as the practice became widespread in the early-2000s, researchers started investigating if this process actually does reduce greenhouse gas emissions when the energy required to transform an ear of corn into ethanol is considered (11). One study found that 29% more energy was consumed to make a gallon of ethanol than it was proposed to have saved, when production of corn, transportation, and fermentation/distillation processes were calculated (12). This neglects the negative impact of soil erosion, pesticide use, and algal (and cyanobacterial) blooms caused by agricultural runoff.

Not only is corn a wasteful way to make ethanol, the plant itself is inefficient at

using photosynthesis to convert the Sun's energy to make sugars. Photosynthetic bacteria are approximately ten times more efficient than land plants at capturing the sun's energy and converting it into usable energy, at a rate of approximately 10% (13). *Synechocystis* sp. PCC 6803 is a single-celled photosynthetic bacterium that is being investigated for its usefulness in production and exportation of biofuels, polyesters, and other chemicals from minimal media sources using light, water, and atmospheric carbon dioxide (14–18). All of these chemicals reach concentration at which they are toxic to the cell, inhibiting production, and yet there remain limited studies as to the cellular effects of the each of these. One method to alleviate this toxicity is to increase export rates prior to internal accumulation. In addition, a secreted product is far easier to retrieve than one stuck within the cytoplasm. However there remains little understanding of the many putative proteins responsible for efflux of endogenously produced molecules that are destined for the cell surface or are waste products in cyanobacteria when compared to our knowledge of *Escherichia coli* or *Saccharomyces cerevisiae*. Nor is there much understanding of transportation processes of those chemicals produced by the genetically modified versions of the cyanobacteria. This understanding of chemical transport can have a critical role promoting the efficiency of converting CO_2, water, sunlight, and minerals into producing and purifying industrially relevant chemicals from cyanobacteria.

1.2 Dissertation goals and outline

There is a common theme throughout the chapter to identify phenotypes conferred by varying native and exogenous genes that encode resistance-nodulation-family (RND) transport proteins. The cell wall, with its many transport proteins and porins, is a critical

feature for transporting chemicals in and out of the cell, yet *Synechocystis* sp. PCC 6803 possesses a variety of little-explored mechanisms that this research aims to elucidate throughout the following chapters. To identify the native mechanisms of *Synechocystis* sp. PCC 6803, SD100, a single-colony isolate of *Synechocystis* sp. PCC 6803 will be used.

I begin the research investigating two putative proteins that have been identified in the plasma membrane of SD100 (19, 20); I want to see if SD100 genes *slr2131* and *sll0180* encode proteins that are homologous to *E. coli* proteins AcrA and AcrB. The *Escherichia coli* proteins AcrA and AcrB, in conjunction with outer membrane duct TolC, are responsible for the efflux of a wide variety of molecules including antibiotics, detergents, free fatty acids (FFAs), and dyes including the namesake of the proteins, acriflavine (21, 22). There has been limited research on the efflux in cyanobacteria of the resistance-nodulation-division (which includes AcrB) family transporters responsible for efflux. Only one study that does more than identify the location of the *Synechocystis* efflux proteins in the membrane by showing that the loss of function of the AcrB homolog hampers efflux of various chemicals (23). I accomplish my goals by creating a suicide vector for each of the genes that I would like to remove, generating four strains based on either SD100 or a previously created high FFA producing variant of SD100, SD277, that have either *slr2131* or *sll0180* exchanged for *aphA*, a gene that confers resistance to kanamycin (Kan), through the process of allelic replacement. The *sll0180* and *slr2131* mutant strains of SD277 possesses significant decreases in the extracellular FFA concentrations and increased intracellular FFA concentrations. Both *sll0180* mutant strains possess lower tolerance to each of the antibiotics and R6G. The *slr2131* mutant

strains are more of a mélange, with varied tolerances to antibiotics and R6G compared to their respective parent strains.

There were a number of strategies applied in this research to accomplish the overarching goals, which is to improve fatty acid accumulation outside of the cyanobacteria cells so that these hydrocarbons can be used in the production of biofuels, while also filling some knowledge gaps with respect to cyanobacterial efflux. Purification of FFAs from inside of the cell is more difficult than that if they are present supernatant; modification of the efflux systems in cyanobacteria could eliminate the necessity for lysing and drying of the cells. This would allow the cells to continue to grow and produce while simply removing FFAs from the growth media. To accomplish this goal, I added genes that encode components of the *E. coli* multidrug efflux pump, AcrAB-TolC by creating a series of expression vectors that were then added to SD100 and a high fatty acid producing strain, SD277. All 10 of these subsequent strains were tested for intracellular and extracellular FFA concentrations. Then, in addition to FFA secretion, I wanted to show that the protein(s) that make up this pump are indeed affecting secretion, so I exposed the cells to a number of antibiotics and the dye, rhodamine 6G (R6G). The added genes again benefitted the cells, but this time both for the SD100 and the SD277-based strains. There is minimal research on synthesizing exogenous membrane transport proteins in cyanobacteria, so this study helps to elucidate the potential wide-ranging uses for such a system.

In the penultimate chapter, the four strains that contained a deletion mutant of *slr2131* or *sll0180* were complemented with the *E. coli* gene homologous to it, *acrB* or *acrA*, respectively. I was particularly interested to see what effects a complementation

experiment would have on the deletion mutants as the mutants had varying phenotypes throughout the first set of experiments, but the mutation predominantly caused decreased tolerance to antibiotics and R6G. Only the tolerance to Cm was restored in the complementation strains, but SD277 with the deletion of *slr2131* and the complementation using the *E. coli acrB* gene had the highest extracellular of FFAs of any strain tested in these experiments, providing evidence that AcrB can move FFAs out of the SD277 cell. The addition of *E. coli acrA*, did not increase extracellular FFA concentrations of the *sll0180* deletion strains significantly, indicating that *E. coli acrA* may not have the adaptor protein in SD100.

My final section concludes the findings of the research and applies the context that the findings of this research can be used in both adding to the tools of using cyanobacteria to produce industrially-relevant chemicals and furthering the understanding of efflux in SD100. I also present the context for biofuel research in the current state of energy technologies.

CHAPTER 2

BACKGROUND: UNDERSTANDING EFFLUX OF *SYNECHOCYSTIS* SP. PCC 6803, ITS HOMOLOGOUS SYSTEMS, AND THE INDUSTRIAL CONTEXT

2.1 *Synechocystis* sp. PCC 6803 as a cell factory

Synechocystis sp. PCC 6803 is a single-celled cyanobacterium with a 3.6Mb genome that is relatively easy to manipulate when compared to eukaryotic algae (24–26). With a cell generation time between 6 and 48 hours, the growth is slower than most conventional bacteria used for genetic studies and production of industrially relevant chemicals (27). For example, *E. coli* is commonly used for bacterial research and chemical production; its generation time is 15-20 min (28). Additionally, unlike the single genome copy of *E. coli*, *Synechocystis* sp. PCC 6803 has between 10-14 copies of its genome (29, 30). Foreign genes must be placed in each copy of the cyanobacterial genome for the alteration to be stable through generations, adding more time for new strain production. While initial generation of cyanobacterial modification are more time-consuming and difficult to achieve than *E. coli*, the cyanobacteria do not require sugars to grow. They instead rely on sunlight, CO_2, water, and basic minerals at low concentrations, making cyanobacteria less expensive to grow when compared to heterotrophic organisms. Additionally, these cyanobacteria grow much faster and at higher concentrations of CO_2 (when submerged in water) than land crops (26). Cyanobacteria are more ideal to use than land crops since they do not require arable land. Idyllically, using cyanobacteria to consume atmospheric CO_2 and using that sequestered atmospheric CO_2 for human energy needs, we can stop removing sequestered carbon from subterranean environments that has led to ever-increasing greenhouse gas

emissions. However, the more energy used to purify the FFAs from cyanobacteria for fuel creation, the further from carbon neutral this process becomes. When producing FFAs with cyanobacteria, 70-80% of the cost deals with harvesting the cells, lysing, drying the products, and extracting the lipids with solvents (14, 31). This can be reduced if some of these steps are removed, namely cell harvest and lysis. In turn, this step reduction saves both energy costs for production and environmental costs by pushing the process towards carbon neutral. Simply secreting the products and collecting the products from the supernatant can achieve this goal.

While emphasis has been given here to FFA production, cyanobacteria have also been engineered to create a number of other biofuels including hydrogen, ethanol, butanol, isoprene hydrocarbons, isobutanol, and fatty alcohols (18, 32–38). Hydrogen production in *Lyngbya aestuarii* BL J, a marine cyanobacteria that resides in mats, was shown to be 45-times greater than *Synechocystis* sp. PCC 6803 at producing hydrogen during a fermentation assay in which the strains were grown in aerobic, illuminated conditions during the day and dark, anoxic conditions at night (32). The *L. aestuarii* BL J did not require any genetic modifications, while the *Synechocystis* cyanobacteria did require the removal of the type I NADPH-dehydrogenase complex for this maximal, yet inferior hydrogen production. *L. aestuarii* BL J resides in microbial mats in which conditions at night quickly become anoxic in the cell-dense growth conditions, creating conditions that facilitate the production of hydrogen though fermentation (33). The following cyanobacteria made for biofuel production were formed using *Synechocystis* sp. PCC 6803 as the parent strain. Ethanol was created by disrupting the poly-β-hydroxybutyrate pathway and inserting alcohol dehydrogenase genes from *Zymomonas*

mobilis, *Anabaena* sp. PCC 7120, and *Synechococcus* sp. PCC 7942 (34). Isoprene hydrocarbons were created by expression of a codon-optimized isoprene synthase from *Peuraria montana* (35). Isobutanol was produced by adding two *Lactococcus lactis* genes, *kivD* and *adhA*, which convert 2-keto acids in alcohols (37). Similar to the creation of ethanol, fatty alcohols production necessitates the disruption of the native glycogen/poly-β-hydroxybutyrate. Copies of the fatty acyl-CoA reductase gene were inserted in its place (38). The strain named SD277 was created to overproduce FFAs by deleting native genes and adding foreign genes. The strain successfully increased production of 12-, 14-, 16-, and 18-carbon saturated fatty acids (14). A further analysis of the strain is included in Chapter 3 due to its pertinence to this research.

Utilization of cyanobacteria for chemical production is not specific to biofuels, as they are currently used for several other products. *Synechocystis* sp. PCC 6803 has been engineered to create 3-hydroxybutyrate (3HB), a precursor to plastics (16). A precursor to synthetic rubbers and polymers, ethylene, was produced in a modified *Synechocystis* sp. PCC 6803 using the expression of the *efe* gene from *Pseudomonas syringae* (39). L-lactate was also produced in *Synechocystis* sp. PCC 6803 using the *Bacillus subtilis ldh* gene (40).

Numerous strains have been created, many of them in using *Synechocystis* sp. PCC 6803 as the parent strain, in order to produce products beneficial to society. They include modifying native genes and introducing exogenous ones. But nearly all removal of genes attempts to affect competing pathways to the pathways that lead to the production of a desired chemical, removal feedback inhibition signals, or the weakening of cell wall components. The genes added encode proteins responsible for producing the product or a

precursor to it. The source of the exogenous genes ranges from other cyanobacterial species to eukaryotic plants. There has been limited research identifying exogenous genes that are added that yield proteins responsible for active efflux of industrially relevant chemicals.

2.2 Limited understanding of efflux in *Synechocystis* sp. PCC 6803

Efflux is a critical consideration when using these cyanobacteria as a production factory. Many of the chemicals that can be produced become toxic when researchers begin removing feedback inhibition loops and adding exogenous genes or upregulating native ones. In fact, the chemicals themselves that are the limiting factors. The production of ethanol in cyanobacteria may be limited by the ethanol produced because of their inherent lack of tolerance to it (41). Numerous research groups have been actively producing ethanol in *Synechococcus* sp. PCC 7942. Researchers have overexpressed an endogenous alcohol dehydrogenase gene, *adhA*, and added an exogenous alcohol dehydrogenase pyruvate decarboxylase from *Zymomonas mobilis* (34, 42). These genetic modifications led to significant increases in ethanol production but raised concerns regarding the cells' tolerance to the chemicals produced in far higher concentration than in a wild-type cell. By removing a number of genes encoding putative transport proteins, one group discovered a function of Slr0982, an ATP-binding cassette (ABC) efflux transporter, which moves ethanol out of the cell (43). With this knowledge of how the bacterium's native mechanism operates, more genetic modifications can be performed to accompany the increased ethanol production in the cell. These include modifying the promoter sequence of *slr0982*, optimizing the codons that encode the protein of Slr0982,

or adding exogenous genes shown to move ethanol out of a bacterial cell. One example is the *FPS1* gene found in *Saccharomyces cerevisiae* that has been shown to limit ethanol concentrations in the yeast cell through ethanol efflux (44).

There are far fewer proteins in *Synechocystis* destined for active membrane transport than in *E. coli,* which possesses 79 ABC family proteins, making up 69 independent systems as some function in multiple structures. Of the 69 proteins, 57 are transporters; there is a limited understanding of the remaining proteins. What is of particular note is that of the 57, 44 are uptake systems while only 13 are efflux systems (45). There are 11 identified ABC transporters in *Synechocystis* sp. PCC 6803. The identified exporters include Evr, Slt, Slr0982, Sll1180, and Slr2019, while the following are ABC importers: Bgt, Urt, Fut, Ggt, Nat, and Mnt (23, 43, 46–52). Instead of having a 3:1 ratio of importers to exporters as in *E. coli*, *Synechocystis* sp. PCC 6803 possesses close to a 1:1 ratio, likely reflecting the necessity for *E. coli* to move more chemicals into their cells, namely sugars for energy. *Synechocystis* does not require uptake of any sugar, but instead small chemicals such as iron (through the Fut transporter), necessary for photosynthesis that yields the sugar a cyanobacterium requires for survival. The ABC exporters of *Synechocystis* sp. PCC 6803 are responsible for efflux of viologen herbicides, phosphatidylglycerol, O-antigen, and lipid A (50, 53).

No small multidrug resistance (SMR) family proteins have been predicted nor observed in *Synechocystis* sp. PCC 6803 (54). Two multi antimicrobial extrusion (MATE) proteins have been predicted and the deletion mutants of *slr0896* and *slr1543*, each produced strains that exhibited increased sensitivity to acriflavine and methyl viologen (53). There exists a total of six open reading frames that have been identified as

the major facilitator superfamily (MFS) transporters. But, only two of the putative MFS proteins, which includes Sll1154 and Slr0616, have been found to exist in the membrane; each is induced by cold-weather, but no functions have been identified (55–57). Researchers were investigating mechanisms of cold resistance in cyanobacteria for an understanding of cold-response in photosynthetic organisms (57). Another efflux system for bacteria is the resistance-nodulation-division (RND) family. There are five pumps belonging to this family that have been predicted in *Synechocystis* sp. PCC 6803, including the Slr2131. Further explanation of the RND pumps and their associated membrane fusion proteins is contained in Chapter 3, but Slr1270 is a *Synechocystis* sp. PCC 6803 membrane channel that is predicted to operate in conjunction with ABC and RND efflux systems to extrude chemicals out of the cell in a similar manner to the function of TolC in *E. coli*—outer membrane duct that allows for passive flow of chemicals out of the cell (58).

The possible role of *slr1270* in *Synechocystis* sp. PCC 6803 was first mentioned in the literature in 2004 (59), but the description of the actual function was not published for a decade. Initially, the Slr1270 protein was determined to be a homolog to the *E. coli* TolC protein by isolating the outer membrane of *Synechocystis* via sucrose density centrifugation and aqueous two-phase partitioning that was then mapped out on 2D-PAGE (60). This proof that Slr1270 is present in the outer membrane of *Synechocystis*, led investigators to then compare the gene sequence to that of TolC and 10/11 peptides matched (59). The gene *slr1270*, was then expressed in *E. coli* in which its structure, size, and function were observed. When studying the conductance of the channel when exposed to KCl, the results actually suggested a larger pore than that of *E. coli* TolC. But,

Slr1270 likely has a similar function based on ion-sensitivity, displaying a potential voltage similar to TolC when exposed to a potassium gradient. The structure is also similar based on the sequence of amino acid residues (60).

In 2015, researchers in Portugal continued this research by investigating the role of Slr1270 in respect to protein and metabolite efflux *in vivo* in *Synechocystis* sp. PCC 6803 (61). This research group removed part of the *slr1270* gene from the *Synechocystis* sp. PCC 6803 genome using a substitution deletion, performing a homologous recombination with a gene cassette conferring resistance to Kan and observed a number of phenotypic alterations. First, the researchers modified the growth conditions, finding that the *slr1270* mutant is more susceptible to colder temperatures and a number of antibiotics. The flasks in which the mutant cells were grown had less accumulation of cyanobacterial cells on the sides of the Erlenmeyer flask in which the bubbles, due to the aeration, were popping, compared to *Synechocystis* sp. PCC 6803. The authors indicated that this lower number of cyanobacterial cells affixed to the Erlenmeyer flask indicated a lower amount of biofilm formation. The modified cells also secreted fewer proteins. The following proteins were not present in the extracellular space of the *slr1270* mutant strain but were present in the extracellular space of *Synechocystis* sp. PCC 6803: Sll0723, Sll1951, Sll1009, and Slr1751. Based on transmission electron microscopy (TEM), the mutants lacking *slr1270* may be lacking the key structures of the S-layer, likely secreted through the outer membrane duct, Slr1270. This research hypothesizes that there are efflux pumps in *Synechocystis* sp. PCC 6803 that use this channel to secrete various chemicals, yet none have been shown to do so.

2.3 The role of multidrug efflux transporters in *Escherichia coli*

E. coli has been a model organism for the better part of its recorded history since its isolation from the human colon (62). It became notable beginning in the 1960s for its ability to survive with a number of dyes and detergents (22, 63) and then later with antibiotics, in which hospital patients were shown to serve as hosts for antibiotic-resistant *E. coli* (64–66). In the early 2000s, research began showing the structures of some of these pumps, promoting the expansion of this research field, which allows a better understanding of these patients' bacteria and for exploration into other uses for these pumps (67, 68).

There are five main types of efflux pathways in bacteria: The ABC transporter family that relies on ATP from ATP hydrolysis for substrate relocation. The other four, include RND, MFS, SMR, and MATE, which rely on a proton gradient across the membrane (69).

The ABC family is a well-studied efflux transporter system found in nearly all branches of life, including humans in which an ABC transporter is located in the blood-brain barrier, often hampering the effects of therapeutics destined for the brain by extruding them (70). *E. coli* possesses examples of each of these efflux pump systems that have a specific function in the cell. *E. coli* uses the BtuCD-F system for Vitamin B_{12} uptake (71), while the MsbA ABC transporter moves lipid molecules from the cytoplasm across the inner membrane (72). EmrD, part of the MFS family, is responsible for the efflux of detergents and carbonyl cyanide *m*-chlorophenylhydrazone, an inhibitor to oxidative phosphorylation (73–75). SMR proteins are usually 100-140 amino acids in length some of which, such as YdgE and YdgF, confer resistance to drugs like ethidium

and methyl viologen (76). MATE pumps are not researched often in *E. coli* other than to use an *E. coli* strain that is hypersusceptible to antimicrobials to investigate the MATE pumps from other bacteria such as the *Staphylococcus aureus* MepA pump (77). While this is not a complete list of transporters in *E. coli*, these reflect some of the more completely studied examples.

The final efflux system, and the one used as a catapult for this research, is the RND system. It uses one outer membrane duct, the membrane-fusion protein (MFP), and the RND efflux pump in the periplasmic membrane (69). With the combination of these three proteins, the cell can launch various substrates through the inner and outer membranes and the periplasm. One common outer membrane duct is TolC of *E. coli* that associates with the MFP, AcrA, and the efflux pump, AcrB (78). For reference please review Figure 2.1. Without any one of these three components, *E. coli* cells become hyper susceptible to a range of antimicrobial agents including antibiotics, dyes, organic solvents, and detergents (63, 79, 80). This type of efflux pump is not uncommon. *Neisseria meningitidis* and *N. gonorrhoeae* have the *farAB* efflux pump responsible for fatty acid resistance as the bacteria colonize the human nasopharynx (81). *Pseudomonas aeruginosa* uses RND pumps to acquire widespread resistance to antibiotics throughout hospitals through hyperexpression of the genes that encode the MexAB-OprM efflux pump, a homolog to the *E. coli* AcrAB-TolC system (82, 83). This research looks to further the understanding of RND transporter in *Synechocystis* sp. PCC 6803 and SD277 in addition to using exogenous *E. coli* transporters in the cyanobacteria.

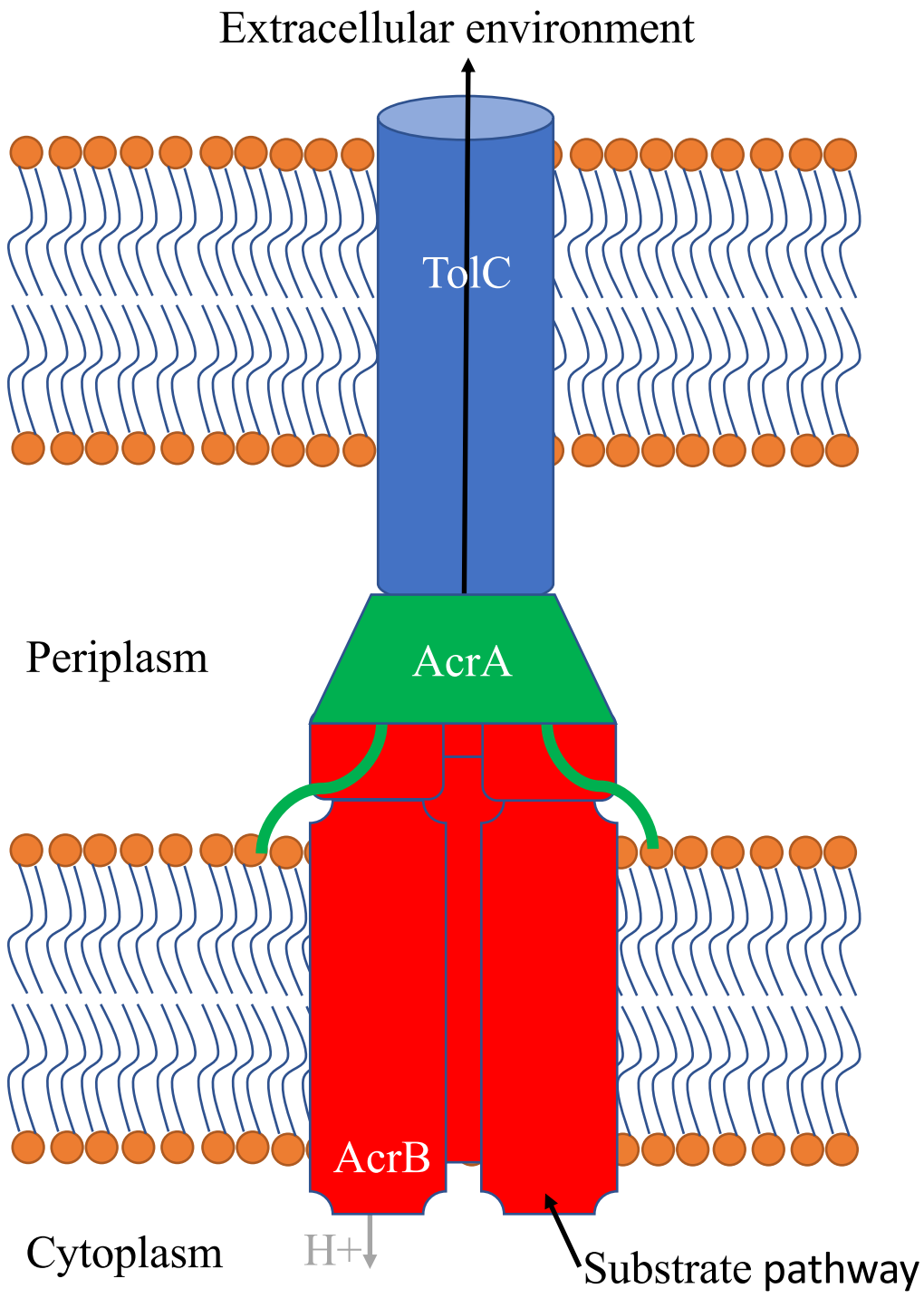


Fig. 2.1 Schematic of the AcrAB-TolC multidrug efflux system as established in *E. coli*. The blue cylinder represents the TolC exit duct. The green trapezoid with two tails represents two AcrA adaptor proteins. The three red rectangles sans corners represent the AcrB homotrimer transporter protein.

2.4 Exogenous transporters synthesized in cyanobacteria

Cyanobacteria have evolved in limited-nutrient conditions, which may be the reason they possess two-fold fewer transport systems relative to genome size when compared to pathogenic bacteria like *Escherichia coli*. However, these two groups of bacteria have a similar number of drug resistance transporters suggesting both need to export toxins from the cells (84). As photoautotrophs, cyanobacteria do not import nutrients in the same quantities as heterotrophic bacteria; therefore, they lack many of import mechanisms that *E. coli* and other heterotrophic bacteria possess.

As discussed in Chapter 2.1, many exogenous genes, including those from other bacteria and eukaryotic plants, have been expressed in *Synechocystis* and other related cyanobacteria for use in chemical production. However, few of these genes additions address active efflux and those that do focus entirely on sugar efflux, predominantly in *Synechococcus elongatus* sp. PCC 7942. Genes encoding transporters from *E. coli* and *Zymomonas mobilis* were expressed in *Synechococcus elongatus* sp. PCC 7942 (85). The GLF protein from *Z. mobilis* allows for diffusion of glucose and fructose across the membrane in or out of the cell without phosphorylation of the sugar. The LldP protein from *E. coli* is classified as a lactate permease protein that facilitates L-lactate transport through a proton/substrate exchange across the membrane, but the LldP protein can also transport D-lactate and glycolate across the membrane (86, 87). The *E. coli* gene *invA* encodes the protein InvA that cleaves sucrose to glucose and fructose. Each of the genes were placed in *Synechococcus elongatus* sp. PCC 7942 and the proteins InvA, GLF, and LldP were successfully synthesized. Fructose and glucose were both successfully made and secreted from the genetically modified cyanobacterial cells that produced proteins

InvA and GLF. This research provides insight into the possibilities of *E. coli* genes being synthesized and having the same function in cyanobacteria that the exogenous proteins had with respect to the transport of sugars through cell membranes in their native *E. coli* bacterium cell.

Multiple examples exist of introducing exogenous sugar transporters into cyanobacteria to promote growth in the dark, which are often unable to simply transport the sugar into the cells. This was shown by adding either glucose transport gene *glut1* from human erythrocytes or *hup1* from *Chlorella kessleri*, an algae, to *Phaeodactylum tricornutumcan* that allowed the organism to grow in the dark (88). Another example of exogenous transport protein synthesis in cyanobacteria also involved *Synechococcus elongatus*. The cyanobacteria grew continuously in diurnal conditions by adding the *galP*, *xylE*, *xylA*, and *xylB* genes from *E. coli* (89). Cyanobacteria do not grow when the sun sets or the incubator lights are switched off, exhibited by a change in gene expression to facilitate respiration and 3-polyhydroxybutryrate (3HB) synthesis that are upregulated at the shift from day to night (90).

These studies elucidate the basis for using *E. coli* transport genes in cyanobacteria to facilitate chemical movement. In *Synechococcus elongatus*, both efflux and uptake of sugars have been demonstrated in cells that could not perform either before the genetic modifications. My research addresses the possibility of using *E. coli* RND transporters to more FFAs out of the cell in cyanobacteria, expanding on the existing body of research using exogenous proteins from *E. coli* in cyanobacteria to transport chemicals while furthering the understanding of the native RND mechanisms in *Synechocystis* sp. PCC 6803.

Chapter 3

REMOVING PUTATIVE TRANSPORT GENES FROM *SYNECHOCYSTIS* SP. PCC 6803 AFFECTS FREE FATTY ACID SECRETION AND TOLERANCE TO ANTIBIOTICS AND A DYE

3.1 Introduction

3.1.1 Overview and hypotheses

The genome of Synechocystis sp. PCC 6803 has been routinely modified for a variety of reasons including the understanding of photosynthesis to the production of ethanol and FFAs for fuel. The high FFA producing strain, SD277, was created to significantly increase the amount of various FFAs produced by a *Synechocystis* sp. PCC 6803 cell when compared to its wild-type parent strain, SD100 (14). In this research, I hypothesized that the FFAs are actively moved out of the cells. However previous research, including the Liu *et al.*, 2011 research article that originally described the creation and testing of SD277, hypothesized that FFAs are transported out of the cell passively. This passive mechanism is described as the "flip-flop" method in which polar intracellular FFAs pass through the lipid bilayer that makes up the membranes (91, 92). The FFA from the lipid membrane is then ejected into the extracellular space. I want to identify possible active mechanisms for the secretion of FFAs and elucidate if those mechanisms are also responsible for tolerance to antibiotics and R6G, a dye shown to be secreted in *E. coli* by the AcrAB-TolC efflux pump.

I hypothesize that removal of the gene that encodes the putative efflux pump, *slr2131*, adversely affects secretion of FFAs and tolerance to antibiotics and R6G. In addition, there are a couple of proteins whose genetic code bear homology to the MFPs of

the RND and ABC efflux systems. In this study, I hypothesize that the removal of the gene that encodes one of those putative MFPs, *sll1080*, will also adversely affect FFA secretion and tolerance to antibiotics and R6G. In order to test my hypotheses, I needed to remove each of the genes from the cyanobacterial cells in both SD100 and SD277. I accomplished this by creating a vector that recombines with the flanking regions beside each of the genes of interest, swapping a nucleotide cassette that confers resistance for Kan in place of the gene of interest. Once these four strains were created, I then performed an assay in which the mutant strains were grown in culture with varied concentrations of three antibiotics and R6G to find the half maximal inhibitory concentration (IC_{50}). In addition, I grew 100mL cultures of all four strains using aeration to promote dense growth that were then used to identify FFAs in the supernatant corresponding to extracellular FFA concentration, and, using the Folch Method, I extracted the FFAs out of the intracellular space to measure the intracellular FFA concentration.

3.1.2 Proteins responsible for RND efflux in cyanobacteria

Based on the nucleotide sequence, there are 7 open reading frames in *Synechocystis* sp. PCC 6803 that may encode RND transporters (54, 93): *slr0369*, *slr0454*, *sll0142*, *sr0794*, *slr6042*, *slr6043*, and *slr2131*. No research has been performed with respect to the proteins encoded by *slr0794*, *slr6042*, or *slr6043*, so their exact functions remain unknown. Of proteins encoded by the aforementioned genes that have been investigated, Sll0142 was shown to be located in the membrane of *Synechocystis* sp. PCC 6803. Strains with a mutation of *slr0369*, in which a gene cassette conferring resistance to Kan was recombined into the middle of *slr0369*, possessed altered pilin

glycosylation and increased susceptibility to sodium dodecyl sulfate (SDS), likely reflecting an efflux mechanism (23). A strain with a mutation of *slr0454* was found to be more susceptible to chloramphenicol (Cm) and is a suspected alter efflux route for the *slr2131* gene product (23). I found that *slr2131* shared significant homology to *E. coli acrB*, as discussed in the following section, and decided to pursue an investigation of it.

The other regularly investigated RND efflux pump has been shown to be used for efflux of copper in *Synechocystis* sp. PCC 6803. The CopRS system modulates copper in the cell as it is necessary for many cellular processes, but can become toxic as the concentrations increase (94–98).

3.1.3 Identifying the homologs of AcrA and AcrB

Numerous studies have identified the location and existence of the proteins Slr2131 and Sll0180 in the cell membrane of *Synechocystis* sp. PCC 6803 (20, 56, 59, 99, 100). I examined the genetic homology of each of the genes encoding proteins Slr2131 and Sll0180, *slr2131* and *sll0180* respectively, to potential homologous genes in *E. coli*. The *Synechocystis* sp. PCC 6803 gene, *slr2131*, has previously been observed to have increased expression when *Synechocystis* sp. PCC 6803 is grown in the presence of hexane (101). Hexane is one of the many substrates of AcrB in *E. coli* (102), so I compared the nucleotide sequence of *slr2131* to *acrB*, the *E. coli* gene encoding the AcrB multidrug efflux pump. The *acrB* gene is located in an operon with *acrA* in the chromosome of *E. coli* so I then compared *sll0180* to *acrA*, the *E. coli* gene encoding the AcrA MFP.

The Slr2131 protein is a homolog to AcrB/AcrD/AcrF family of multidrug efflux pumps (20). Slr2131 is found exclusively in the plasma membrane of *Synechocystis* sp.

PCC 6803 (19). The function of the *E. coli* protein AcrB is understood as the actual pump that expels antibiotics from bacterial cells with the assistance of the adaptor, AcrA, and exit duct, TolC (66). The relationship and interaction between AcrA, AcrB, and TolC is intensively reviewed in Chapter 4, in which I describe the addition of these genes to the various *Synechocystis* strains. Current research findings based on the genetic sequence analysis and location of each protein provide some insight as to the function of *Synechocystis* sp. PCC 6803 proteins Slr2131 and Sll0180, but my research looks to find phenotypic differences in strains lacking the native genes that encode these two proteins. The *Synechocystis* sp. PCC 6803 gene, *sll0180*, is the least studied of the three deleted genes in this work. Only a genetic analysis and protein localization of Sll0180 has been completed, in which is it hypothesized that Sll0180 is one of four predicted MFPs responsible for anchoring Slr1270 to the cell membrane (59). Slr1270, as highlighted in the previous chapter, is a homolog to *E. coli* TolC, the outer membrane duct that allows passage of the substrates of the AcrB pump through the outer membrane. Sll0180 is one of two predicted MFPs that is a lipoprotein, with the other being Sll1053 (20, 99). AcrA is also a lipoprotein, anchored to the inner membrane using a lipid moiety further pointing to the conclusion that AcrA and Sll0180 have the same function in their respective organisms.

The nucleotide sequence homology between *slr2131* and *acrB* is a 55% identity with 10% of the Needleman-Wunsch alignment requiring gaps to facilitate maximal homology (103). The two genes only vary by 36 nucleotides in length, in which *acrB* consists of 3150 and *slr2131* has 3186. This is strikingly similar when one considers that *Escherichia* and *Synechocystis* genera diverged evolutionarily somewhere between 3.1

and 3.254 billion years ago (104–107), an especially long time ago considering the oldest known cyanobacteria fossils are 3.5 billion years old (1). This divergence can be compared to that of *Escherichia* coli and closely related *Salmonella enterica*, diverged 49 to 166 million years ago (107–109). The closest matching gene to the *E. coli acrB* in *Salmonella enterica* is also called *acrB* and shares almost complete homology with an identical length of 2150 nucleotides in which 86% of the nucleotides matched with virtually no gaps required for this alignment.

There were two genes considered for removal to identify a potential RND MFP, *sll1053* and *sll0180*. At the time of deciding, there was no research identifying the function of either, so I made the determination based on a BLAST search of similar sequences in the SD100 chromosome to *E. coli acrA* in which I quickly narrowed down the search to *sll1053* and *sll0180*. Both were about 25% longer than the 1194 nucleotide sequence of *E. coli acrA*, and the aligned sequences had evenly spaced gaps. The sequence identity of *sll1053* and *acrA* is 46% with 28% of the alignment requiring gaps to facilitate the homology. The other potential homolog of *E. coli acrA* is SD100 *sll0180*, which had a sequence homology that was also 46% when compared to *E. coli acrA* but had slightly fewer gaps required to align the sequences at 25%. So, then I was curious exactly how conserved are the sequences of these two cyanobacterial genes. When *sll1053* is compared directly to *sll0180*, there is only a 51% identity with 18% of the nucleotides representing gaps. If you compare the sequences of *acrA* to another *E. coli* membrane fusion protein responsible for an RND efflux pump, *acrE*, they are far more homologous with 67% identity and only 5% sequence needing gaps to perfect the alignment and a total nucleotide length within 33 base pairs of each other, 1155 bp length

of *E. coli acrE* compared to 1188 of *acrA*.

When comparing the protein sequences of the native *Synechocystis* sp. PCC 6803 proteins to the homologs in *E. coli*, AcrA is created from 397 amino acids while Sll0180 is composed of 501 amino acids. The first 131 amino acids of Sll0180 share an 18% identity to AcrA and, similarly, nearly the last 262 amino acids of Sll0180 share a 19% identity to AcrA (Sll0180 amino acids 500 and 501 were not included in this identity analysis) (110). But this leaves the gap from amino acids 132 to 237 in Sll0180, bearing virtually no homology to AcrA. AcrB share far more protein sequence homology to Slr2131 relative to the comparison between AcrA and Sll0180. Slr2131 has a protein sequence length of 1061 amino acids, while AcrB has 1049 amino acids. Amino acid 2 through 1017 of Slr2131 watched matched with the a 1016 amino acid length central region of AcrB for a 41% identity with no gaps in the alignment (110), indicating that the vast majority of the two proteins sequences share protein binding sites and transmembrane domains that a paramount to the functioning of each pump.

3.1.4 The high fatty acid producing *Synechocystis* strain

SD277 is a strain derived from SD100 (a single colony isolate of *Synechocystis* sp. PCC 6803), previously created with a series of genetic deletions and additions that dramatically increase the levels of FFA production (14). In order to survive, SD277 has an ability to release FFA. However, a clear mechanism for release has yet to be demonstrated, while the authors present the idea of a "flip-flop" method for release, but there is no empirical evidence that this is the case in SD277 FFA production. In the first series of modifications, the *Synechocystis* 6803 gene *slr1609* was removed and replaced with the *E. coli* gene *tesA* lacking its signal sequence (*'tesA*), to trap the enzyme in the

cytosol and prevent it from moving to its normal location in the periplasm. In *E. coli*, this had the effect of increased accumulation of many of the FFA, some by as much as 26 times (111). Slr1609 is an acyl-acyl carrier protein (ACP) synthetase, while TesA is an acyl-ACP thioesterase. Slr1609 takes FFA and creates Acyl-ACP, while TesA takes Acyl-ACP and creates FFA, a more desirable outcome for the production of FFA. Some of the modifications to create SD277 include the following gene additions that resulted in proteins producing the listed saturated free fatty acids: Cc *fatB1* (accumulates myristic acid), Uc *fatB1* (accumulates lauric acid), and Ch *fatB2* (accumulates octanoic and decanoic acids). Each encode for ACP thioesterases from *Cinnamomum camphorum*, *Umbellularia californica* and *Cuphea hookeriana*, respectively. In addition, *slr1951* and *slr1710* were deleted to weaken the cell membrane to promote for FFA release. The following genes were also removed from the SD100 chromosome because the proteins encoded by each of the genes is responsible for competing with the FFA synthesis pathway and therefore were an impediment to creating a high FFA producing SD100 strain: *slr2132, slr1993, slr1994, slr2001,* and *slr2002*. I hypothesize that an active mechanism to rid the cell of the FFA will lead to a higher concentration of FFA outside of the cell.

3.2 Materials and Methods

3.2.1 Growth and culture conditions

A single cyanobacterial colony is picked by a sterilized loop from a BG-11 media plate that has been streaked using cells from -80^{o}C frozen stock and used to inoculate a BG-11 medium plate, a universal media for growing cyanobacteria (112), and grown with

illumination at 30°C in an environmental chamber (SANYO, Osaka, Japan). If the selection or maintenance of plasmid-containing cyanobacteria cells or a cyanobacterial strain containing a substitution deletion was necessary, the appropriate antibiotic was added at concentrations of 50 μg/mL of Kan and 30 μg/mL of streptomycin (Str) and spectinomcin (Spec). The cells were then added to a 1-mL modified BG-11 medium buffered by 10 mM N-[tris(hydroxymethyl) methyl]-2-aminoethanesulfonic acid (TES) NaOH (pH 8.2) in a glass test tube. The tube is incubated with illumination and intermittent shaking for 2–4 d. Once these starter cultures reach an optical density at 730 nanometer wavelength (OD_{730}) of 0.6 as measured by a spectrophotometer (Thermo Fisher GENESYS 10S, Waltham, MA), the samples were transferred to a 10 mL BG-11 medium culture grown in a 250-mL Erlenmeyer flask with aeration at 100 mL/min. This protocol uses TES buffer and air aeration to keep the pH around 8 at the beginning inoculation stages to minimize the lag phase. Once the culture reaches a mid-log level growth phase, the culture of cyanobacterial can be used to test the IC_{50} to Amp, Cm, Ery, and R6G, using a 1:2 dilution to have a starting OD_{730} of 0.4. If the culture is to be used for FFA analysis, 20 mL of BG-11 media without TES is added to the existing 10mL culture, as TES negatively affects FFA concentration (14). This now 30 mL culture is grown to a concentration of 8-10x10^8 cells/mL and then subjected to analysis of intracellular and extracellular FFA concentrations.

3.2.2 Construction of vectors for gene deletion

Table 3.1 Primers used to delete *slr2131* and *sll0180* from SD100 and SD277

Primer Name	Sequence	Reference
A 3Z slr 2131 end F	GAG AGA GCT CGA CCC CGT TGT GTT AA	this research
A 3Z slr 2131 center R	GAT TTA TTT TCT TGG ATC CCA TTA CGG CCG ACA TTG TTA CAT ATC T	this research
A 3Z sll 0180 end F	CCT GGA GCT CTT GAC AAT GAC GAC AAT C	this research
A 3Z sll 0180 center R	TTT TTA GTT CTG GAT CCC ATT ACG GCC GTA GTT AAT TGA CTC A	this research
B 3Z slr 2131 center F	TGT AAC AAT GTC GGC CGT AAT AGG ATC CAA GAA AAT AAA TCT GGT CTT ATT	this research
B 3Z slr 2131 end R	GCG CGT CTA GAT ATC CCA GTT CCA TTC TTT G	this research
B 3Z sll 0180 center F	TGA GTC AAT TAA CTA CGG CCG TTG GAT CCA GAG AAC TAA AAA GTC TA	this research
B 3Z sll 0180 end R	CGG GTC TAG ACT GCT GGG CAT TAT C	this research
Kan BamHI	CAT TAC ACC AAG GAA TTA GGA TCC GTC GAC C	this research
EagI SacB	ATA TCG GCC GGA ACA TCG ACA AAT ACA TAA GGA AT	this research
2131 w/in A	CCA CTT CTT TGG TAT TGA TGG CAG	this research
2131 w/in B	GGA GCT TGG ATA ATG GTG ATG AAA TAA	this research

Table 3.2 List of Plasmids Used to Delete *Synechocystis* genes

Plasmid Name	Description	Parent	Curtiss Collec.	Reference
pGEM-3Z	commercially available cloning vector	N/A	pGEM-3Z	
pPSBA2KS	Vector that harbors *sacB*-KanR cassette	N/A	pPSBA2KS	(20)
pΨ694	pGEM-3Z harboring *slr2131*flanking regions with *sacB*-Kan^R cassette inserted between	pGEM-3Z	pΨ694	this research
pΨ695	pGEM-3Z harboring *sll0180* flanking regions with *sacB*-Kan^R cassette inserted between	pGEM-3Z	pΨ695	this research
pSpec	Expression vector conferring resistance to Str and Spec	RSF1010	pΨ568	this research
pKan	Expression vector conferring resistance to Kan	RSF1010	pΨ687	this research
p*acrB*	Expression vector encoding *E. coli acrB*	pΨ568	pΨ691	this research
p*acrA*	Expression vector encoding *E. coli acrA*	pΨ687	pΨ692	this research
p*tolC*	Expression vector encoding *E. coli tolC*	pΨ568	pΨ699	this research

To remove a gene from SD100 or SD277, it must be removed from all 10-14 copies of the genome (29). This can be accomplished using the cyanobacterium's natural uptake of plasmids, swapping the gene of interest for a cassette that confers resistance to Kan. This allows the newly transformed cells to be selected by growth on Kan-containing media. To allow this transformation, I created two plasmids: one to delete *slr2131* and another to delete *sll0180*. I will use *sll0180* removal as the example, but the exact same process is performed for creation of the plasmid to remove *slr2131*, but with regions respective to *sll0180*. Two 700 base pair regions located immediately upstream and immediately downstream of *sll0180* were amplified using iProof™ High-Fidelity PCR Kit (Bio-Rad, Hercules, CA, USA) from *Synechocystis* sp. PCC 6803 using oligonucleotide primers that I designed (Table 3.1). The "A 3Z sll 0180 end F" and "A 3Z sll 0180 center R" primers amplified the upstream regions of *sll0180*, while the "B 3Z sll 0180 end R" and "B sll 0180 center F" primers amplified the downstream region. The primers (IDT, Coralville, IA, USA) contain a DNA sequence that places distinct restriction enzyme cleavage sites flanking *sll0180*. One restriction enzyme cleavage site was placed at the ends of the flanking regions furthest from *sll0180*, NotI (upstream) and SphI (downstream). Two restriction enzyme cleavage sites were placed in the end of each flanking region nearest to *sll0180*, BamHI and EagI, which is the same for each flanking region. The first reason for placing the same two restriction enzyme cleavage sites at the end of the flanking region closest to *sll0180* was to allow the two independently amplified flanking regions to be digested by the same restriction enzyme so that the flanking regions from either the upstream or downstream ends of *sll0180* can be ligated together. Once each flanking region was amplified using a colony PCR of SD100, then

each DNA fragment is digested with restriction enzyme BamHI. Using the same BamHI site for each of the flanking regions allows the two fragments of DNA to be ligated together. Then a newly created 1.4kb DNA sequence, containing the upstream region attached directly to the downstream region in their native origin, now lacks *sll0180* in the middle. This was then digested again at either end of the 1.4kb sequence using restriction enzymes NotI and SphI to allow the 1.4kb fragment to be ligated into the multiple cloning region of high copy number vector pGEM-3Z (Promega, Madison, WI, USA) that was also digested with NotI and SphI restriction enzymes using 1 μL the DNA ligase enzyme and 1 μL of DNA ligase buffer in a 10 μL reaction volume (NEB, Ipswich, MA). The vector was then electroporated into *E. coli* Top10 cells (Life Technologies, Carlsbad, CA, USA) and then the cells were plated on LB with ampicillin (Amp) at a concentration of 50 μg/mL and grown overnight at 37ºC in lysogeny broth (LB) using the Miller modification (113) and supplemented with 1g/L of glucose (all remaining reference to LB media hereafter are using the Miller recipe with added glucose). Individual colonies were analyzed for the presence of the 1.4kb region of ligated flanking regions using a colony polymerase chain reaction (PCR) with the GoTaq reaction mixture (Promega, Madison, WI) and the outermost "end" primers, in this case: A 3Z sll 0180 end F and B 3Z sll 0180 end R (Table 3.1). The thermocycler temperature and time condition were as follows: Initial denaturation for 2 min at 95ºC, then the next 3 conditions were repeated 30 times of denaturation for 1 min at 95ºC, annealing for 1 min at 56ºC, and extension for 1.5 min at 72ºC, and finally, the final extension step occurs for 7 min at 72ºC. Those primers amplified the 1.4kb sequence in pGEM-3Z, if it existed, but did not amplify any sequence in the *E. coli* Top10 genome. Now the reason for having two restriction enzymes

cleavage sites between the now ligated flanking regions from the upstream and downstream regions of *sll0180* and is to force the direction that the *sacB*-Kan^R cassette was placed between the flanking regions. One end of the *sacB*-Kan^R cassette has the NdeI cleavage site, while the other end has the KpnI cleavage site based on the primers used to amplify the cassette: Kan BamHI and EagI SacB (Table 3.1). The *sacB*-Kan^R gene cassette was amplified from pPSBA2KS, obtained from the Vermaas group (114), plasmid DNA. The two cleavage sites, BamHI and EagI, were digested using corresponding restriction enzymes concurrently in both pGEM-3Z bearing ligated flanking regions of *sll0180*. Then the ligation of the *sacB*-Kan^R cassette between the upstream and downstream fragments of *sll0180* occurred using the T4 DNA ligase enzyme (NEB). This vector was electroporated into *E. coli* Top10 cells (Life Technologies) and colonies containing this Kan-resistance conferring plasmid was identified using colony PCR amplification of the *E. coli* colonies using the GoTaq reaction mixture and the same primers used when identifying the presence of the 1.4kb flanking region in pGEM-3Z; however, in this instance the region is now 4.6kb with the addition of the of the 1.4kb flanking region to the 3.2kb *sacB*-Kan^R cassette so the annealing time increases from 1.5 min to 4.5 min. Once identified, the colony containing the now identified pψ695 plasmid was grown overnight in liquid LB media at 37^{o}C. The following morning, the replicated plasmid is isolated from the *E. coli* cells using a Miniprep Plasmid Purification Kit (Qiagen, Hilden, Germany). The plasmids are summarized in Table 3.2 and stored at -20^{o}C until they are needed. The identical process was completed to create pψ694 with the exception of using the flanking regions of *slr2131* and corresponding 4 primers (A 3Z slr 2131 end F, A 3Z slr 2131 center R, B 3Z

slr 2131 end R, and B slr 2131 center F) instead of the *sll0180* flanking regions and its corresponding primers.

3.2.3 Strain construction using transformation

Table 3.3 List of Strains Used to Delete *Synechocystis* genes

Strain Name	Description	Parent	Curtiss Collec.	Reference
SD100	*Synechocystis* sp. PCC 6803 single colony isolate	*Synechocystis* sp. PCC 6803	SD100	(115)
SD277	High free fatty acid producing strain	SD100	SD277	(14)
SD100 Δ*slr2131*	SD100 with Δ*slr2131* :: sacB-Kan^R	SD100	SD659	this research
SD277 Δ*slr2131*	SD277 with Δ*slr2131* :: sacB-Kan^R	SD100	SD660	this research
SD100 Δ*sll0180*	SD100 with Δ*sll0180* :: sacB-Kan^R	SD277	SD661	this research
SD277 Δ*sll0180*	SD277 with Δ*sll0180* :: sacB-Kan^R	SD277	SD662	this research

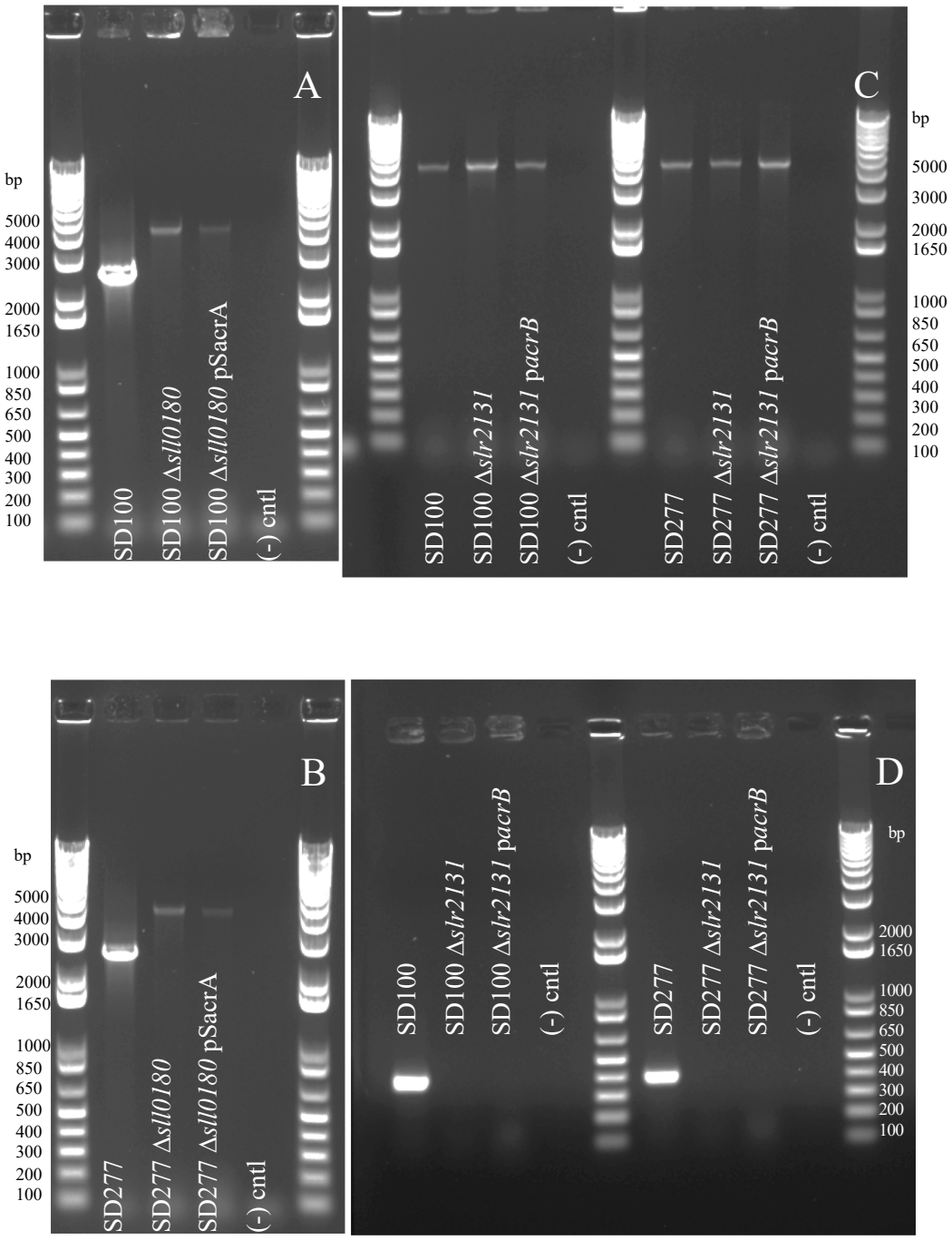


Figure 3.1 Electrophoresis gel displaying the DNA fragments created from PCR of using primers of either end fragments of *sll0180* in (A) SD100, SD100 Δ*sll0180*, SD100 Δ*sll0180* p*SacrA* and (B) SD277, SD277 Δ*sll0180*, SD277 Δ*sll0180* p*SacrA.* Electrophoresis gel displaying the DNA fragments created from PCR using primers of either end fragments of *slr2131* in (C) SD100, SD100 Δ*slr2131*, SD100 Δ*slr2131* p*acrB*, SD277, SD277 Δ*slr2131*, and SD277 Δ*slr2131* p*acrB*. Electrophoresis gel displaying the DNA fragments created from PCR of using primers of a region within *slr2131* in (D) SD100, SD100 Δ*slr2131*, SD100 Δ*slr2131* p*acrB*, SD277, SD277 Δ*slr2131*, and SD277 Δ*slr2131* p*acrB*.

Versions of the transformation process have been widely used since it was first published in 1982 (116). 400ng of pψ695 was added of 17µL of SD100 at a concentration of $1x10^6$ cells/mL. This combination incubates in the illuminated incubator (SANYO) for 4-5 hours and then was diluted with 50µL of BG-11 liquid media (112), which was then plated onto a nitrocellulose membrane that was placed on BG-11 media. Once a visible light green patch of cyanobacteria develops, the nitrocellulose membrane was moved to a BG-11 plate with 50µg/mL of kanamycin sulfate (Sigma Aldrich, St. Louis, MO) in an effort to exchange the *sacB*-Kan^R cassette for every *sll0180* gene in all 10 to 14 chromosome copies in each cell. To identify if complete segregation has occurred, the primers that encode either end of the flanking regions of *sll0180*, A 3Z sll0180 end F and B 3Z sll0180 end R (Table 3.1), were used with GoTaq PCR reaction and template DNA from the colony that was now growing on a BG-11 with Kan media plate. The template DNA was obtained by performing a freeze/thaw (-80ºC to 55ºC) cycle in triplicate of the cyanobacteria sample. In Figure 3.1, the first lane of each image is the DNA ladder. The ladder is repeated throughout the gels. In Fig. 3.1A, lane 2 in the SD100 native gene control of *sll0180* and lanes 3 and 4 are SD100 Δ*sll0180* and SD100 Δ*sll0180* p*acrA*, respectively, with the A 3Z sll 0180 end F and B 3Z sll 0180 end R primers used in the PCR reaction mixture. The primers will work with the other components of the reaction mixture to amplify the end each of the flanking regions surrounding *sll0180* and amplify a ~2,900bp native region or a ~3600bp region that contains the *sacB*-Kan^R cassette. Important in lanes 3 and 4, there are amplified sequences at ~2,900bp indicating these two strains no longer contain the native *sll0180* gene. In Figure 3.1B, the same intentions of identifying the deletion of *sll0180* from strains exist except in SD277 derivatives. In

Figure 3.1C, A 3Z slr 2131 end F and B 3Z slr 2131 end R primers were used to identify that a ~3,000bp region is present between the 1400bp flanking regions; unfortunately, the length of the *sacB*-Kan^R cassette and native *slr2131* are nearly the same length. So, all Figure 3.1C shows is that either the *sacB*-Kan^R cassette or the native *slr2131* gene is located between the flanking regions surrounding *slr2131*. So in Figure 3.1D, I used a different set of primers that only identified the presence ~400bp native nucleotide sequence of in the center of the *slr2131* gene, 2131 w/in A and 2131 w/in B. The results of primers specific to the ~400bp nucleotide sequence for SD100, SD277, SD100 Δ*slr2131*, SD100 Δ*slr2131* p*acrB*, SD277 Δ*slr2131* p*acrB*, and SD277 Δ*slr2131* can be seen in Figure 3.1D in which there is no amplified DNA fragment present in any of the deletion or complementation strains, while the control strains for each of those, SD100 and SD277, using the same primers, has the native *slr2131* fragment amplified. Each of the negative control, marked as "(-) cntl," lanes have just dH_2O instead of a cyanobacterial template. The four strains are referred to throughout this dissertation as: SD100 Δ*slr2131*, SD100 Δ*sll0180*, SD277 Δ*sll0180*, and SD277 Δ*slr2131*. All of these strains are summarized in Table 3.3 using primers highlighted in Table 3.1.

3.2.4 Tolerance assays to determine half maximal inhibitory concentrations (IC_{50})

Cyanobacterial strains were grown to mid-log level in BG-11 liquid media (and antibiotic if needed). The cyanobacteria were then diluted to half of their current OD_{730} (0.35 to 0.4) into 96-well plates (Corning, Corning, NY, USA) containing a concentration from 10ng/mL to 10μg/mL of either Amp, Cm, R6G, or erythromycin (Ery) to identify the relative IC_{50} of each strain. The optical density at 790 nm wavelength of each well of the plate was read using a plate reader (BioTek, Winooski, VT, USA) initially to verify

that each well was in the appropriate density range and then again after 3 days of growth at 30°C on a shaker operating at 60 rotations per minute to determine the IC_{50}. The only IC_{50} that was not determined was for Amp as every strain grew uninhibited at a concentration of 10μg/mL, so they were subjected to concentrations up to 10mg/mL to determine the IC_{50}. The optical density measurements were input into software that allows for the production of a non-linear regression curve using the log of the chemical concentration versus the cell concentration in 4-parameter variable slope model (Prism GraphPad, La Jolla, CA, USA). This identified the IC_{50} as well as the 95% confidence interval of that concentration.

3.2.5 FFA separation and gas chromatography measurement

Each of the six strains used in this chapter are grown to between $8x10^8$ and $1.1x10^9$ cells/mL in BG-11 liquid media using aeration with humidified, filtered air. 20mL of the culture was removed and centrifuged at 6000 x g in an Oak Ridge Centrifuge Tube. The entirety of the supernatant was removed and placed into a new Oak Ridge Centrifuge Tube. The cell pellet had the intracellular FFA extracted separated from the extracellular FFA using a modified Folch Method (117, 118). The Folch method consists of the cell pellet was resuspended in 3 mL of a ratio of 2:1 of chloroform:methanol mixture, which was vortexed at room temperature for 24 h at low speed. The solvent and resuspended pellet mixture was filtered using a PTFE membrane and the resulting solvent was then evaporated using a positive nitrogen evaporator (N-EVAP 111, Organomation, Berlin, MA) until the sample was dry. To begin processing of both samples (119), the Folch extracted sample and the supernatant sample, 10 mL of hexane and 0.2 mL of H_3PO_4 were added as a method of direct transesterification. The tubes were then placed on a

tabletop orbital shaker at 180 rpm for 30 min at 37°C and then centrifuged at 6000 x g for 5 min. 1mL of the uppermost layer (hexane layer) was removed and transferred to a glass tube (13 x 100mm) and dried on a nitrogen evaporator. The dried sample was then dissolved in 1mL of hexane. Then the dissolved sample was analyzed by a gas chromatograph (GC) (Shimadzu GC 2010) with a Supelco Nukol capillary column (30m x 0.53mm x0.5μm) and flame ionization detector (119). GC operating conditions were as follows: split ratio 1∶5; inject volume 1 μL; nitrogen carrier gas with constant flow rate 30 mL⁄min; H_2 40 mL⁄min, air 400 mL⁄min, make up gas (nitrogen) 5 mL⁄min; injector and detector temperature 250°C; and oven temperature started at 100°C and increased at a rate of 10°C⁄min to 220°C and held for 10 min. Each FFA compound was identified by comparing its retention time with that of a standard of lauric acid, myristic acid, palmitic acid, and stearic acid (Sigma Aldrich, St. Louis, MO, USA). Compound concentrations in samples were quantified based on the area under the chromatogram peak in comparison with the standards. Each sample was run through the gas chromatograph in triplicate and the concentration of each FFA present was averaged for the three data points. Each strain undergoes this process in triplicate. The four aforementioned FFAs were selected for two reasons: the previous research describing the FFA concentrations produced by SD277 almost entirely consistent of these four chemicals and the genetic modifications that exist in the chromosome of SD277 were performed to create lauric acid, myristic acid, palmitic acid, and stearic acid. I searched for a number of other saturated and unsaturated fatty acids but was never able to measure any besides the four measured.

3.3 Results and Discussion

3.3.1 Mutants have decreased tolerance to most chemical treatments

Table 3.4 IC_{50} of mutant strains to Ampicillin, Chloramphenicol, Erythromycin, and Rhodamine 6G in which IC_{50} of derivative strain is significantly different than respective parent strain unless otherwise indicated with ns for not significant.

	SD100	SD100 Δ*slr2131*	SD100 Δ*sll0180*	SD277	SD277 Δ*slr2131*	SD277 Δ*sll0180*
Treatment	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL
Amp	955.2 ± 256	1280 ± 230 (ns)	598 ± 296	1570 ± 170	1001 ± 120	450 ± 119
R6G	6.08 ± .871	4.03 ± 0.43	4.61 ± 0.51	1.41 ± 0.12	2.59 ± 0.12	0.94 ± 0.27
Cm	2.290 ± 0.401	0.586 ± 0.142	1.442 ± 0.206	4.749 ± 1.043	1.873 ± 0.354	1.085 ± 0.589
Ery	0.358 ± 0.081	0.054 ± 0.002	0.172 ± 0.1	0.427 ± 0.249	0.171 ± 0.044	0.034 ± 0.007

Significant increases in tolerance are highlighted in green.

The *Synechocystis* genes, *slr2131* and *sll0180,* were deleted from all 10 to 14 chromosome copies of both SD100 and SD277, which were verified through colony PCR (Figure 3.1). Then each of the four strains now containing a gene deletion were exposed to variety of concentrations across multiple log levels of Amp, Cm, Ery, and R6G. The IC_{50} of SD100 Δ*sll0180* to Amp, Cm, Ery, and R6G is significantly decreased, with percent differences of 37%, 37%, 52%, and 12%, respectively, when compared to SD100 (Table 3.4). A similar, if not more dramatic pattern, is observed in SD277 Δ*sll0180*, which possessed a significant decrease of IC_{50} to Amp, Cm, Ery, and R6G with percent differences of 71%, 77%, 92%, and 33%, respectively, when compared to SD277 (Table 3.4). This follows a similar pattern to another study that inserted a mutation into *sll0180* rendering *sll0180* useless in its function to encode Sll0180, in which the researchers showed that the mutant strain had an increased susceptibility to SDS, which has been shown to be secreted by the AcrAB-TolC pump in multiple species of bacteria (23, 120, 121). If Sll0180 is required to stabilize the interaction between Slr1270, the outer membrane duct, and Slr2131, the multidrug efflux pump, as is the role of an MFP, then the lack of the MFP decreases the ability of the cell to move pump substrates to the outer membrane duct; and these sets of data highlight the consistent function of Sll0180 with that of an MFP.

The results from the two strains that lacked *slr2131* are more ambiguous (Table 3.2). SD100 Δ*slr2131* possessed a significant decreased tolerance to Cm, Ery, and R6G at 74%, 85%, and 33%, respectively, when compared to SD100 (Table 3.4). However, when SD100 Δ*slr2131* was treated with increasing concentrations of Amp, it was not significantly different than wild-type SD100 (Table 3.4). SD277 Δ*slr2131* had a

significant decrease of tolerance to Amp, Cm, and Ery with percent decreases of 36%, 61%, and 60%, respectively, when compared to SD277 (Table 3.4). Interestingly, SD277 Δ*slr2131* had an 84% increase in tolerance to R6G when compared to SD277 (Table 3.4). The various modifications to the cell wall of SD277 may prevent R6G from entering the cell, but the *slr2131* deletion must work in concert with one of the existing SD277 mutations to the cell membrane structure as SD277 Δ*sll0180* possesses the expected decrease of tolerance to R6G when compared to SD277.

To initially generate SD277, the penicillin-binding protein 2 (PBP2) was removed from the cyanobacteria. In *Staphylococcus aureus*, the modification of the gene that encodes PBP2 has been shown to increase the minimum inhibitory concentration (MIC) of the bacteria when exposed to ceftizoxime, a beta-lactam antibiotic, by way of other spontaneous mutations that modify the peptidoglycan cell layer cross linking (122). SD277 also has a modified cell wall, losing *sll1951*, which encodes an S-layer protein. SD277 has a 50% greater IC_{50} to Amp when compared to SD100 (Table 3.4), Amp may not be able to diffuse into SD277 as readily as it does into SD100. This may be due to the cell membrane modification in SD277. R6G tolerance in SD277 increases when a putative multidrug efflux pump, Slr2131, is removed. This was the only case in all of the mutant strains were a deletion caused an increase in tolerance to one of the chemicals tested. And again, some of the cell membrane modifications may alter the diffusion of the chemicals into the cell, but in this case specifically, the lack of Slr2131 may modify R6G movement into the cell not observed in either SD277 or SD277 Δ*sll0180* as both had a significantly decreased IC_{50} to R6G when compared to SD277 Δ*slr2131* (Table 3.4). I am not sure what mechanism would hold R6G out of the cell, but the location of R6G could

be identified readily as R6G fluoresces when exposed to a laser beam at wavelength 590 nm (123).

The removal of *slr2131* from SD277 may have changed the composition of chemical pumps in the membrane. R6G does not have a similar function to the antibiotics. R6G does not act on ribosomes or prevent cell wall synthesis like many antibiotics. In fact, it has been shown that rhodamine 123, a related dye, can impede ATPase activity in the mitochondria of rats (124). If this same event occurs in cyanobacteria, then ABC transporters, of which *Synechocystis* has many described ABC import mechanisms (125–129), can be affected. These ABC mechanisms all rely on ATPase activity so the removal of *slr2131* may have affected the composition or location of ABC transporters. At lower concentrations, one of these native ABC transporters is unable to pump R6G into the cell due to R6G itself, impeding ATPase activity. This then can cause an increased resistance to the dye (130). While I admit this inhibition of ATPase activity acting on ABC importers may be far-fetched, the idea that the removal of proteins found in the membrane of cyanobacteria causes widespread effects has been seen previously.

Synechocystis sp. PCC 6803 was shown previously to have 20-fold lower permeability to small carbohydrates, inorganic ions, and amino acids when compared to *E. coli* (131). This lack of permeability is likely due to the lack of natural porins in the outer membrane of *Synechocystis* sp. PCC 6803. A recent study hypothesized that the most abundant outer membrane proteins of *Synechocystis* sp. PCC 6803, Slr1841, Slr1908, and Slr0042 that make up 80% of the proteins in the outer membrane, are primarily responsible for allowing diffusion of inorganic ions into the cell (131). These

three proteins are homologs to *Synechococcus* PCC 6301 proteins, SomA and SomB, beta-barrels that allow diffusion and may link the outer membrane to peptidoglycan layer (132). The cell needs these ions for basic function; iron is used in the electron transport apparatus (133). In *E. coli*, for example, Amp uses the outer membrane protein F (OmpF), a diffusion porin, to navigate its way into the cell (134, 135) at which point it interacts with transpeptidase enzyme, inhibiting cell wall synthesis and causing cell lysis (136). Without the movement of these ions through the membrane proteins the cells struggle to survive, but antibiotics use these same porins to move into the cell, so identifying which *Synechocystis* sp. PCC 6803 porins antibiotics or dyes use to get into the cyanobacterial cell will help to uncover the full picture of chemical transport. Amp and other antibiotics are designed to be lethal to bacteria but increasing the production of chemicals like FFAs can also lead to cell lysis or death if intracellular concentrations exceeds the cell tolerance, which may be driven active transport of FFA out of the cell. Understanding the flow of chemicals in and out of the *Synechocystis* sp. PCC 6803 cell is paramount to expanding the biotechnology applications of it.

3.3.2 Extracellular and intracellular FFA concentrations are modified in the four mutant strains when compared to respective parent strains.

Figure 3.2 Extracellular Free Fatty Acid Concentrations of the SD277-Based Deletion Strains where ** is p < 0.005 and **** p < 0.0001

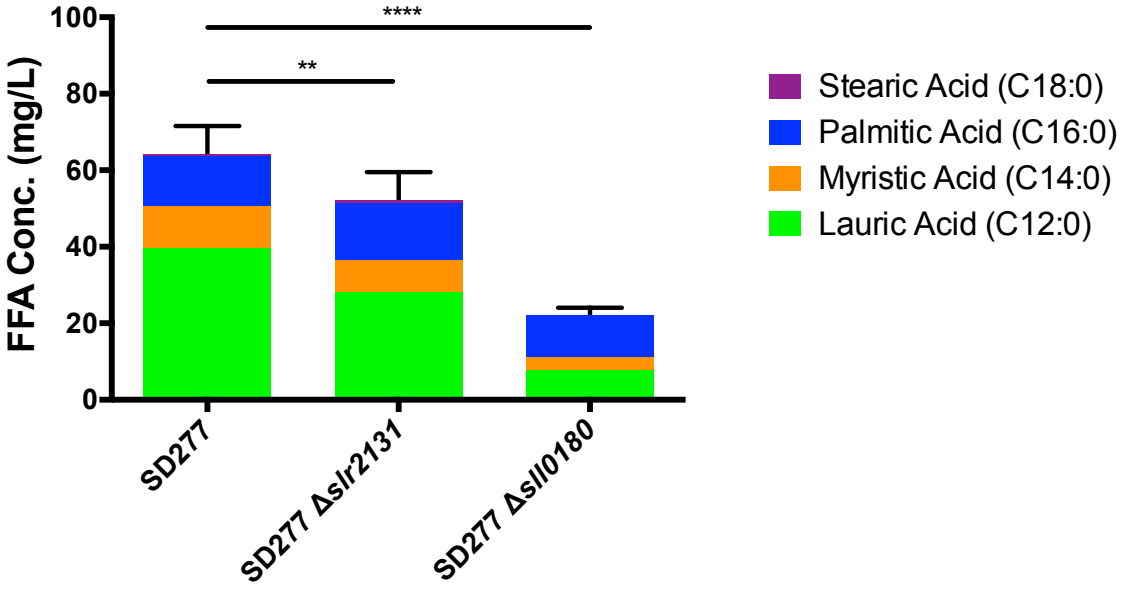


Figure 3.3 Intracellular Free Fatty Acid Concentrations of the SD277-Based Deletion Strains where **** is p < 0.0001 and ns is not signficant.

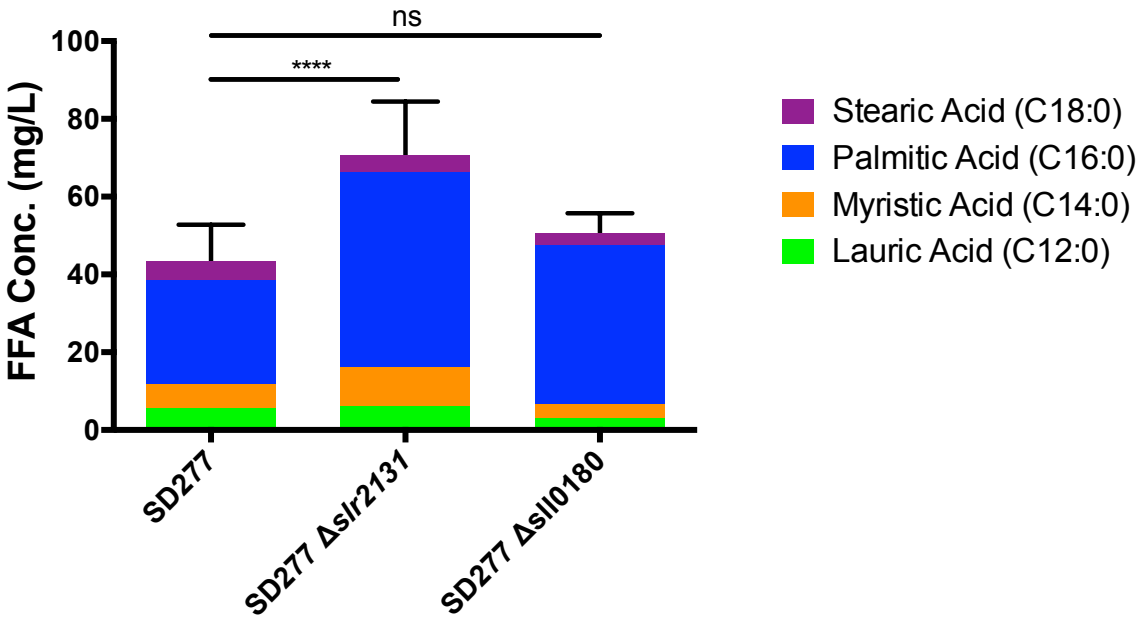


Figure 3.4 Extracellular Free Fatty Acid Concentrations of the SD100-Based Deletion Strains where * is $p < 0.05$ and ** is $p < 0.005$.

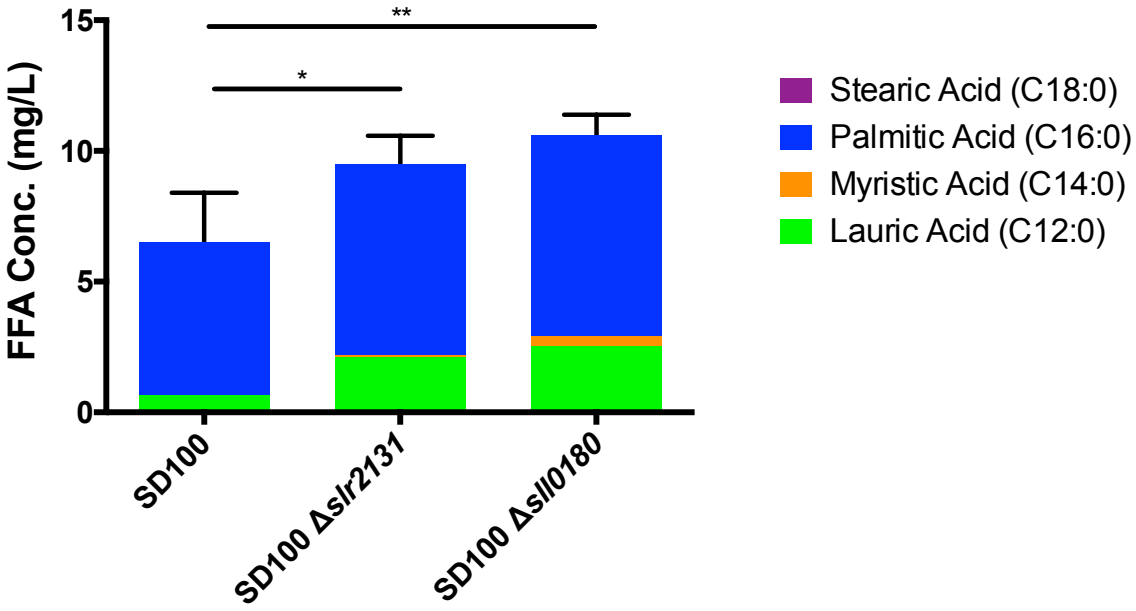
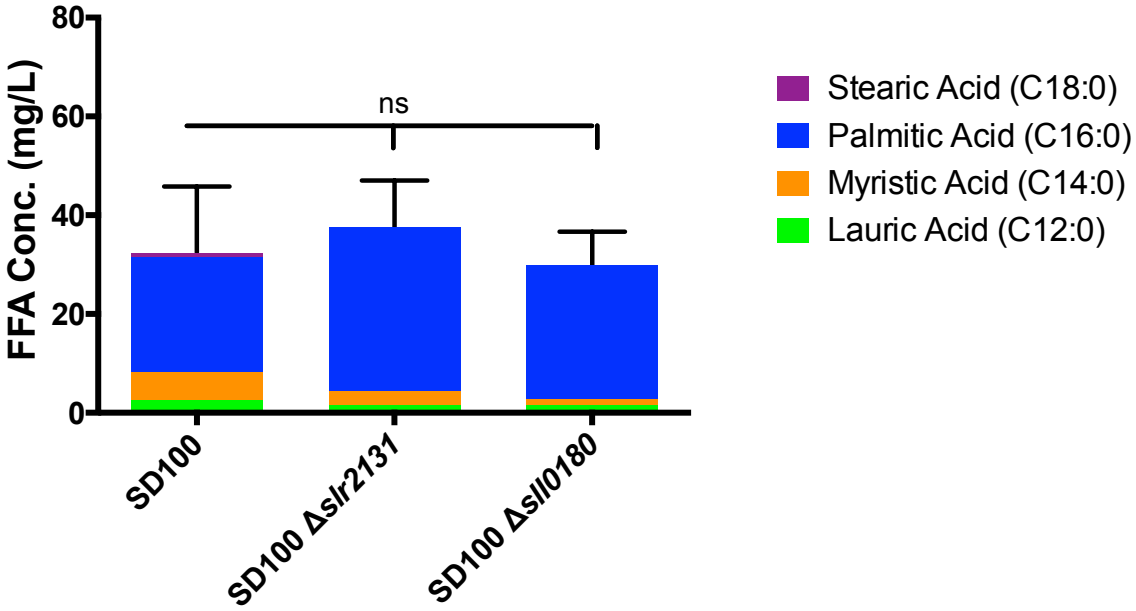


Figure 3.5 Intracellular Free Fatty Acid Concentrations of the SD100-Based Deletion Strains where ns is not signficant



SD277 Δ*slr2131* and SD277 Δ*slr0180* presented a significant drop of extracellular FFA concentrations when compared to SD277 (Figure 3.2), in which all cells are grown to final concentrations $8x10^8$ to $1x10^9$ cells/mL under the same lighting, temperature, and aeration conditions. This corroborates my hypothesis that the proteins encoded by *sll0180* and *slr2131* affect the ability of SD277 to secrete FFA and the movement of FFA out of the cell is not entirely reliant on passive diffusion. In *E. coli*, FFA secretion is mediated by the AcrAB-TolC pump. In *E. coli*, a mutant strain that has modifications of the *acrAB* and *emrAB* (encodes two proteins with similar function to *acrAB*) operons inhibiting synthesis of the encoded proteins exhibited significantly less FFA secretion, but this secretion function is restored when the mutant strain was complemented with a plasmid containing *acrAB* (21). Of note, when there was just a single mutation of either *acrAB* or *emrAB*, the mutant *E. coli* strain did not a possess a significant decrease in extracellular FFA concentrations. Researchers hypothesized that the proteins encoded by the *emrAB* and *acrAB* operons have overlapping functions that can complement one-another with respect to FFA efflux. There has not been any published research to determine if the removal of *acrA* or *acrB* independently of each other will cause the same phenotype with respect to FFA efflux in *E. coli*. In *E. coli*, the *acrA* and *acrB* are located in a 5kb operon together facilitating the previously developed *acrAB E. coli* double mutant, unlike *slr2131* and *sll0180*, which are not even in the same orientation in the genome of *Synechocystis* sp. PCC 6803 let alone in an operon together. The *emrAB* and the *mdtAB* efflux pumps were also removed independently from *E. coli*, but this combination deletion had less of an effect on FFA efflux in *E. coli* than the *acrAB*/*emrAB* double operon mutant strain (21). SD277 Δ*slr2131* had significantly greater intracellular FFA

concentration while SD277 Δ*sll0180* did not have a different intracellular FFA concentration when compared to SD277 (Figure 3.3). SD277 Δ*slr2131* had a significant decrease in extracellular FFA concentration (Figure 3.2) and a significant increase in intracellular FFA concentration (Figure 3.3) relative to SD277 and, taken in combination, this data suggests that the FFAs remained within the cell membrane of the bacteria when *slr2131* was removed from SD277. This adds further evidence to the hypothesis that *slr2131* encodes a multidrug efflux pump that has been previously presented (23). The *sll0180* deletion in SD277 also significantly decreased extracellular FFA concentration (Figure 3.2), and the unchanged intracellular FFA concentration (Figure 3.3) relative to SD277 further provides evidence that *sll0180* encodes an MFP. But besides identifying the roles of the proteins encoded by *slr2131* and *sll0180* there are a couple of other issues that were addressed in the SD277 deletion mutants. The first is that the presence of the *sacB*-Kan^{R} cassette in the genome of SD277 does not hamper the FFA production as intracellular FFA concentration is either unchanged or increased in the two SD277 mutants relative to SD277 (Figure 3.3). In addition, the lack of either of *sll0180* or *slr2131* genes does not hamper intracellular FFA concentration, so the proteins encoded by *slr2131* and *sll0180* do not have a function with any feedback inhibition mechanisms that would work to counteract fatty acid synthesis. Fatty acid synthesis remains as high or higher intracellularly in the two mutant strains when compared to SD277 (Figure 3.3), which is important in attempting to identify proteins with roles in efflux. If either of the SD277 mutants had a decreased intracellular FFA concentration when compared to SD277, then the FFA concentration extracellularly would be irrelevant as the lack of the gene would have a direct impact on fatty acid synthesis and the impacts of the

extracellular FFA concentration could be due to a decrease of FFA production, not efflux. Another possible confounding issue is that the SD277 mutants grew at a different rate than SD277, varying FFA efflux. According to Figure 3.6 and the corresponding statistical tests explained in Chapter 3.2.1, there were no growth rate differences among SD277 and its two mutant strains.

The results with the SD100 mutant strains are more difficult to arrive at conclusions regarding extracellular and intracellular FFA concentrations because both mutants produced greater FFA concentrations extracellularly but had no change with respect to intracellular FFA concentrations when compared to SD100 (Figure 3.4 and Figure 3.5). I do not know why these two deletion mutant strains would have significantly increased extracellular FFA concentrations, but the actual average values of the differences between the mutants and SD100 are between 3 to 4 mg/L. This is a log level lower than the differences displayed in the SD277 mutants when compared to SD277 (Figure 3.2 and Figure 3.3) . So, SD100 may just simply not make a high enough concentration of FFA in these in-lab, 24 h illuminated growth conditions to further our understanding of FFA transportation out of the cyanobacterial cell. SD100 does not make a habit of releasing FFA or other energy storage chemicals to its surroundings in which the cyanobacteria have to rely on stored energy when the sun sets, as do nearly all photosynthetic organisms. The intracellular FFA concentration of the SD100 mutants is unchanged from the SD100 parent strain (Figure 3.5), so neither mutation causes an increase of intracellular FFA concentration, as was seen in SD277 Δ*slr2131* (Figure 3.3), but again in these 24 h illuminated conditions at the optimal temperature with the perfect balance of nutrients and pH, SD100 does not need to store energy for respiration at nighttime in the

form of FFAs, as it can make it readily 24 h per day. The two SD100 mutants do not have a significantly different growth pattern when compared to SD100 (Figure 3.7), illustrating that these two mutations do not have any effect on the growth pattern of SD100 or SD277.

Figure 3.6 Growth Curve of SD277-Based Deletion Strains

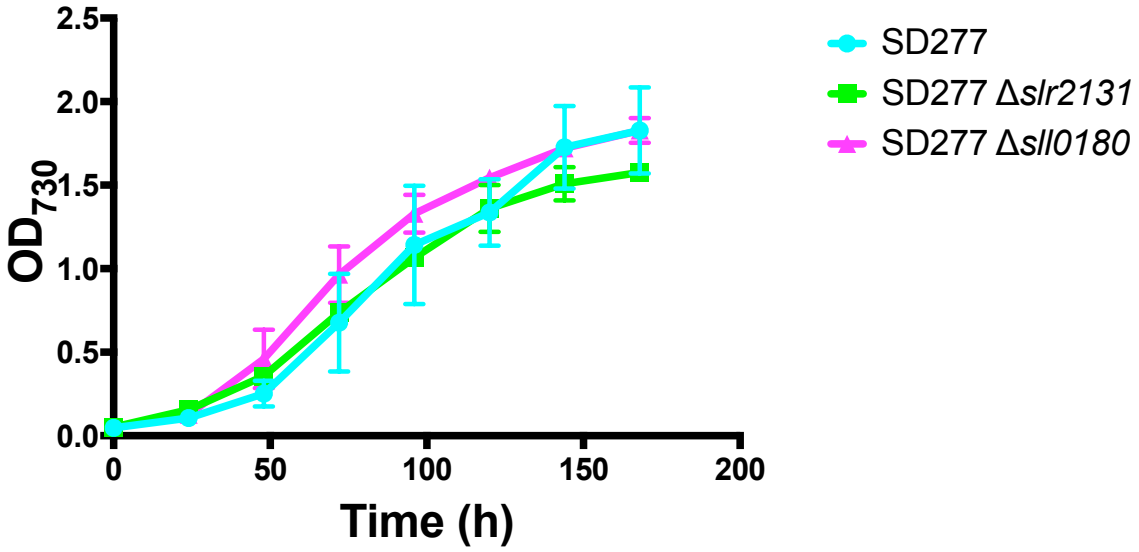
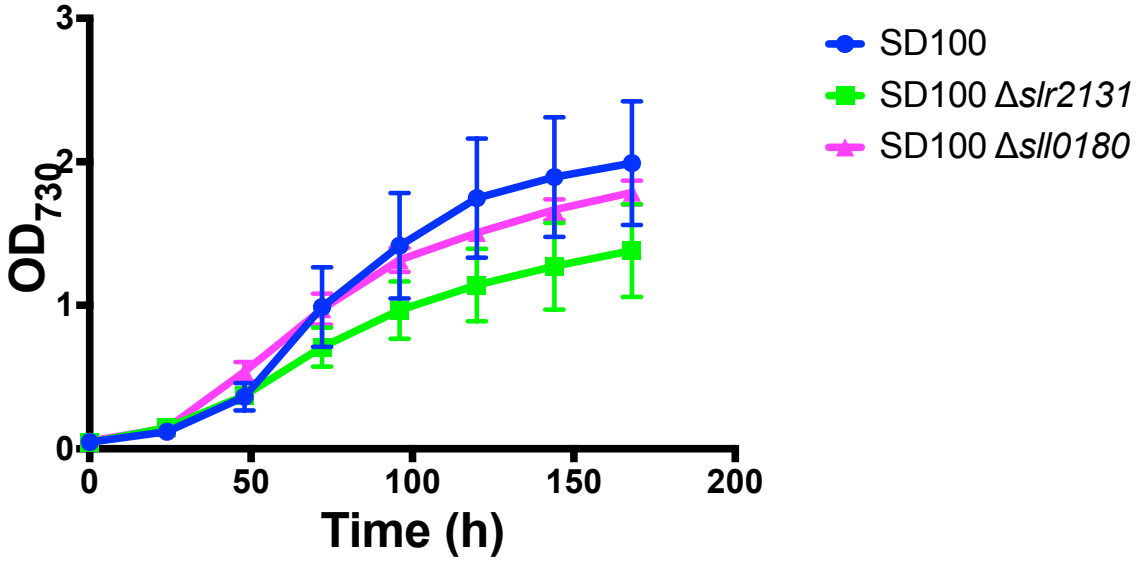


Figure 3.7 Growth Curve of SD100-Based Deletion Strains



3.4 Conclusions

Mutations in *slr2131* and *sll0180* in both SD100 and SD277 effectively decrease tolerance to antibiotics and R6G, without affecting the growth patterns relative to the parent strains (Table 3.4). This is consistent with the hypothesis that *sll0180* and *slr2131* encoding an MFP and multidrug efflux pump, respectively. The only exception to this conclusion was the tolerance of SD277 Δ*slr2131* to R6G increased when compared to SD277. I am not certain why this was the case. The SD100 Δ*slr2131* mutant has decreased tolerance to R6G, so modifications specific to SD277 in concert with the *slr2131* mutation may affect how R6G was also able to penetrate the SD277 Δ*slr2131* cells, decreasing the rate of R6G diffusion into the cell compared to SD277. The lack of the S-layer protein, Sll1951, or the lack of penicillin binding protein 2 (PBP2) may have altered the cell wall of the cyanobacteria in a way to slow down the diffusion of R6G into the cell, without affecting the antibiotics. But the lack of *slr2131* must also affect the progression of R6G into the cell in a manner unlike the SD277 strain lacking *sll0180*.

In the high FFA producing, SD277, both proteins encoded by *sll0180* and *slr2131*, Sll0180 and Slr2131 are necessary for efficient FFA efflux to the extracellular growth medium. Without either of the genes encoding Sll0180 and Slr2131, the concentration of all the tested extracellular FFAs decreases (Figure 3.2) while the concentration of intracellular FFAs increases or remains the same as SD277 (Figure 3.3), indicating that the decrease in extracellular FFA is not due to a decreased production of FFA. In addition, growth rates remain consistent, so the decreases are also not due to a slower growth rate.

These results when taken together present a perspective on the roles that the Sll0180 and Slr2131 proteins have in chemical tolerance and FFA secretion through efflux of these chemicals. My research has illustrated that *Synechocystis* sp. PCC 6803 cells use an active transportation mechanism similar to *E. coli* to move FFA and other chemicals out of the cell. This is amazing as these two genera separated over 3 billion years ago and each has maintained a similar efflux mechanism. The next step in the process of understanding the native efflux mechanism of *Synechocystis* sp. PCC 6803 and possibility of added exogenous mechanisms is to add the genes encoding the AcrAB-TolC pump to increase the tolerance of SD100 and SD277 to the toxic chemicals and promote secretion of FFAs in SD277 and SD100, just as adding a plasmid containing *acrA* and *acrB* promoted FFA efflux in an *acrAB*/*emrAB* mutant *E. coli* strain (21).

CHAPTER 4

THE ADDITION OF *ESCERICHIA COLI* RESISTANCE-NODULATION-DIVISION FAMILY TRANSPORTERS MODIFIES FREE FATTY ACID SECRETION AND TOLERANCE TO ANTIBIOTICS AND A DYE

4.1 Introduction

4.1.1 Overview and hypotheses

This research is an effort to limit the processing costs of producing these precursors to biofuels and to enhance the total concentration of the FFAs through the addition of exogenous genes expressed in SD277 and SD100. *E. coli* genes encoding proteins responsible for sugar transport have been synthesized in *Synechococcus elongatus*, a closely related cyanobacteria to SD100. The synthesized proteins allowed for transport of glucose and lactose in and out of the cell in an effort to permit the cells to grow using these sugars at night instead of the stored sugars produced using photosynthesis. This chapter looks at the use of the AcrAB-TolC efflux pump from *E. coli* to export various chemicals including intracellularly produced FFAs from SD277 and SD100. No research has demonstrated that these *E. coli* transport proteins can be synthesized in any cyanobacterial species. My goal was to increase the extracellular FFA concentration of SD277 and demonstrate, in SD277 and SD100, that these proteins can increase the tolerance of the cyanobacteria to Amp, Cm, Ery, and R6G.

The first hypothesis I test is that adding the gene(s) that encodes individual components of the *E. coli* AcrAB-TolC RND efflux system to SD100 or SD277 will increase the tolerance to various antibiotics and R6G. The vectors and strains will be briefly discussed here with a more comprehensive explanation of their creation in

Chapter 4.2.2. I began with two distinct plasmids each with genetic sequences based on the RSF1010 plasmid featuring *repA*, *repB*, and *repC* that each encode a protein that promotes appropriate replication in the cell and a Ptrc promoter for constitutive expression of the gene ligated downstream of the promoter (137, 138). The only difference between the two different RSF1010-based vectors used in this research, pKan and pSpec (Appendix A.2), is which an antibiotic-resistance conferring cassette is present in the plasmid nucleotide sequence, Kan and Spec, respectively. I created strains that would eventually be used as controls in which I added pKan and pSpec separately and together to SD100 and SD277 to create the following strains: SD100 pKan, SD100 pSpec, SD277 pKan, SD277 pSpec, SD100 pSpec pKan, and SD277 pSpec pKan. Then the three *E. coli* genes used in this research, *acrA*, *acrB*, and *tolC* were ligated into pKan and pSpec. More specifically, the pSpec plasmid was in which *E. coli acrB*, *acrA* and *tolC* genes were located, separately, to create p*acrB*, p*SacrA* and p*tolC*. The pKan plasmid was in which *E. coli acrA* was located to create p*acrA*. I test my hypothesis that adding genes encoding components of the *E. coli* AcrAB-TolC efflux system to SD100 and SD277 will increase tolerance to toxic chemicals and will increase the extracellular FFA concentrations by individually adding each of the following *E. coli* plasmids to both SD100 and SD277: p*acrA*, p*acrB*, and p*tolC*. This created the following six strains: SD100 p*acrA*, SD100 p*acrB*, SD100 p*tolC,* SD277 p*acrA*, SD277 p*acrB*, and SD277 p*tolC*. Then, I added the combinations of *E. coli* p*acrA* and p*acrB* together and, separately, added the combination of *E. coli acrA* and *tolC* together to both SD100 and SD277 to create the following four strains: SD100 p*acrA* p*acrB,* SD277 p*acrA* p*acrB*, SD100 p*acrA* p*tolC*, and SD277 p*acrA* p*tolC*. I was never able to isolate strains with

acrB and *tolC*, nor was I able to isolate mutants with all 3 of the genes together in either SD100 or SD277. I did not see any significant absolute increase in tolerance to any antibiotic with the exception of SD100 possessing p*acrA* and p*tolC*. I did see a significant increase in tolerance of many derivative strains of SD277 to the R6G, but those effects appear to be due to the addition of the plasmid itself as the strains containing plasmid controls also possessed significantly increased tolerance to R6G relative to R6G.

The second hypothesis I test is that adding the gene(s) that encodes individual components of the *E. coli* AcrAB-TolC RND efflux system to SD277 and SD100 will increase the extracellular FFA concentrations in each of the subsequence strains but will not change the concentration of intracellular FFAs relative to the respective parent strains but will increase the extracellular FFA concentrations. This is achieved using the same strains described above but the cells are grown to late-log phase and the supernatant is separated from the cells to isolate the FFAs that have been secreted and the remaining pelleted cells have the FFAs extracted using the Folch Method (118). None of the extracellular FFA concentrations of the derivative strains of SD277 increased significantly compared to SD277, but when compared to respective control strains nearly all the strains expressing two *E. coli* genes simultaneously did have increased extracellular FFA concentrations relative to the SD277 pKan pSpec control strain. Many of the plasmid control vectors possessed a significant decrease in extracellular FFA concentrations. The derivative strains of SD100 had far less variation of FFA concentrations when compared to the FFA concentration results of SD277 and its derivatives.

4.1.2 The *E. coli* AcrAB-TolC efflux pump

AcrAB-TolC is a routinely studied multidrug efflux pump in *E. coli*. It removes a plethora of components from the cells, namely antibiotics, dyes, FFAs, and detergents. Acriflavine resistance protein B (AcrB) is part of the efflux mechanism of *E. coli*. As a member of the resistance-nodulation-division (RND) superfamily, it functions as a proton/substrate antiporter. AcrB is also considered part of a subdivision of RND pumps called Hydrophobe/amphiphile efflux-1 (HAE1), which has been identified to export numerous drugs, dyes, and detergents including acriflavine, hexane, cyclohexane, Cm, Ery, and R6G in *E. coli* (22, 63, 80, 139, 140). Most importantly for this research, the system is also involved in FFA efflux (21). The pump of the system, AcrB, has three monomers, each of which has 1049 amino acid residues that together form a trimer to span the inner membrane, stabilized via connecting loops. On the periplasmic side, AcrB maintains a TolC docking domain and on the opposite side, it possesses a porter domain. The PN1 subdomains from each of the three monomers align to form a pore that is too small for drugs to pass through. On the opposite site of the monomer, the PC1 and PC2 domains allow AcrA to dock in a cleft (67). The 397 amino acid residues of AcrA role is likely maintaining the interaction of AcrB and TolC so the complex can span the cell envelope (141).

The monomers of AcrB can take any of three conformations that have been termed loose (L), tight (T), and open (O) (142). A substrate binds at the loose conformation, then it changes to tight, and finally releases the substrate in the open conformation to the waiting channel of TolC that is stabilized by AcrA (143, 144). As opposed to ATP synthase, AcrB has a functional rotation, not a physical rotation of monomers (145, 146). The three subunits of AcrB do not move in the membrane but instead change

conformation, opening and closing of different pockets in the protein to move substrates from the cytoplasm to the opening of the outer membrane duct (147).

Recognized substrates, such as R6G, will bind to the loose conformation of one of the AcrB monomers and that monomer changes to the tight conformation. This conformational change of an AcrB monomer does not require energy. Once one substrate binds to an AcrB monomer in the loose conformation, a second substrate must bind to a different L monomer before the T monomer (of the initial bound substrate) can release the chemical via the O monomer. The change from T to O is an energy dependent step, relying on ATP hydrolysis and proton uptake from the periplasm (Figure 2.1) (142).

TolC is an outer membrane duct. It is anchored to the outer membrane via a β-barrel, projecting from the outer membrane down into the periplasm (148, 149). It has a 100Å pore which functions as the exit duct for the AcrB transporter (150). AcrB has limited contact with TolC, so AcrA adaptor function is necessary for efficient transfer between TolC and AcrB (Figure 2.1) (151).

AcrA mediates the interaction between AcrB and TolC. AcrA (150) is 210Å long allowing it to span the periplasm (152). This span facilitates the connection between AcrB and TolC (153). The N-terminal of AcrA anchors the protein to the inner membrane, while also binding to AcrB that is lodged in the inner membrane. This combination of interactions stabilizes the AcrA to AcrB connection (Figure 2.1).

4.1.3 The stresses endured by overproducing FFA

When *E. coli* has been genetically modified to produce substantial quantities of FFAs, the cells suffer tremendous physiological stresses. Some of these modifications include overexpression of acyl-ACP thioesterases and acetyl-CoA carboxylase (ACC)

and/or the deletion of *fadD* or *fadE*, that work in the opposite direction of thioesterases (31, 154–156). In these modified strains, cells undergo tremendous inner membrane stress and altered cell morphology due to the membrane change, leading to an 85% reduction in viable cell count (157).

FFA production in *Synechococcus elongatus* also leads to physiological stresses in the cells. In a *Synechococcus elongatus* PCC 7942 strain that possessed similar mutations to SD277 in an effort by researchers to create a high FFA-producing strain, the modified *Synechococcus elongatus* PCC 7942 possessed a series of issues including chlorophyll-*a* degradation and changes to localization of phycocyanin and allophycocyanin, indicating a stress response in the cells. There are also effects to the lipid composition of the membrane (56, 158). The result of this was a decreased rate of cell growth, which may be alleviated by an active efflux mechanism in the cell to extrude FFA.

In the FFA-producing strain, SD277, that was used as a starting point for the research in this study, there was lower cell damage in SD277 cells and an increase growth rate when compared to SD100. So, with respect to the SD277 cells, I was not concerned about the various stresses on the cells as they had not been observed, but I was interested in how the FFAs were secreted for reason highlighted in the following section.

4.1.4 Natural lipid secretion in cyanobacteria

Cyanobacteria possess mechanisms to move lipids out of the cell, yet the mechanisms are not well elucidated. The first measurement of lipid movement was published in regard to *Synechococcus* sp. PCC 7942, in which fatty acids were shown to move across the thylakoid membrane via diffusion (159). FFA acids are also better able to diffuse through the membrane of *Synechococcus* sp. PCC 7002 when compared to

Synechococcus elongatus because of the elevated amount of polyunsaturated FFA that promote diffusion (56, 160). FFAs were hypothesized to be moved across the phospholipid bi-layer using the "flip-flop" method (14), in which the fatty acids diffuse across the membrane where the polar group is supposed to cross through the hydrophobic core of the membrane layer, but this rate may be as slow as one molecule per 24-hour period due to the energy required for this movement (91, 92).

In the previously published research centered on the construction of SD277, the added thioesterases synthesized a significant increase of FFA concentration. However, the previous research did not separate the supernatant from the growing cells before direct transesterification using hexane and phosphoric acid. Instead, researchers relied on previous research indicating that hexane and phosphoric acid will not extract FFA from within the cell (117). My research looks to identify FFAs located in the supernatant and see if the same pattern of FFAs occurs in the supernatant alone. Then the intracellular FFA concentrations are also observed.

4.2 Materials and Methods

Separate cloning vectors are conjugated into SD100 and SD277. One vector, pKan, uses encodes a protein that confers resistance to Kan. The other, pSpec, confers resistance to Str and Spec. Initially *E. coli* genes *acrA*, *acrB*, and *tolC* are placed in SD100 and SD277 individually using the aforementioned expression vectors. Then two vectors each harboring one of the three genes encoding *acrA*, *acrB*, and *tolC* are placed in the strains in different combinations. The only combination that was not successfully created was *acrB* with *tolC.* In addition, a strain in which all 3 genes were placed in either SD100 or

SD277 at the same time was never isolated. These 10 new strains were then used to test for survivability on BG-11 plates containing various concentrations of the dye R6G and Amp, Cm, and Ery.

4.2.1 Growth and culture condition

The growth and culture collection condition are identical to those explained in Chapter 3.2.1; however, if the culture was used for mRNA analysis to identify the expression of exogenous *E. coli* genes in SD100 and SD277 derivatives, another 70mL of BG-11 was added to the 30mL culture that was started from a single colony to create a 100mL culture in a 250 mL Erlenmeyer flask. Once the culture reached the mid-log level phase (OD_{730} of 0.7 to 0.8), two 40mL samples are removed and analyzed for the presence of specific mRNA sequences. One sample identified the presence of the *E. coli* gene of interest, while the other sample identified a constitutively expressed *Synechocystis* sp. PCC 6803 gene. For these experiments I chose *petB* as explained in Chapter 4.2.4.

4.2.2 Construction of expression vector

Table 4.1 Primers used to add *E. coli* genes SD100 and SD277

Primer Name	Sequence	Reference
5' AcrB F	CGA TGG ATC CAG TCT TAA CTT AAA CAG GAG C	this research
3' AcrB R	AAT TGC ATG CAT AAA AAA GGC CGC TTA CGC	this research
3' AcrA R	ATA TGC ATG CAC GGC TCC TGT TTA AGT TAA	this research
5' AcrA F	GCACGGATCC TTACATATGAACAAAAACAGAGG	this research
5' TolCR	GCATGCCATATGTCAGTTACGGAAAGGGTTATGA	this research
3' TolCF	GCATGCGGATCCTTGCGGTAGCGGATTCTGCTA	this research
568F	GCTAGACAGGCTTATCTTGGAC	this research
568R	CGTTGACGAGTATTACCCG	this research
687F	TCC TAT GGA ACT GCC TCG GTG AG	this research
687R	CGG GAT CGG GAT ACT GGT CGT TAC	this research

Table 4.2 List of Plasmids used to Add *E. coli* Genes to SD100 and SD277

Plasmid Name	Description	Parent	Curtiss Collec.	Reference
pSpec	Expression vector conferring resistance to Str and Spec	RSF1010	p⋃568	this research
pKan	Expression vector conferring resistance to Kan	RSF1010	p⋃687	this research
p*acrB*	Expression vector *E. coli acrB*	p⋃568	p⋃691	this research
p*acrA*	Expression vector *E. coli acrA*	p⋃687	p⋃692	this research
p*tolC*	Expression vector *E. coli tolC*	p⋃568	p⋃699	this research
pRL443	Helper plasmid with methylases	RP4	pRL443	(161)
pRL528	Conjugative plasmid	pRL542	pRL528	(161)

The plasmids created for the research described in this chapter are summarized in Table 4.2 using primers summarized in Table 4.1. Two vectors that are based on the RSF1010 multicopy plasmid containing the *repABC* operon were the basis for the addition of each of the *E. coli* genes and also represented controls when each of the plasmids were added to SD100 and SD277 separately and together. The Ptrc promoter, used to constitutively express the downstream gene because is not efficiently repressed by SD100 due to the absence of the LacI repressor (162). Ptrc is also located upstream of the multiple cloning site region on every plasmid (Figure A.2). None of the native promoters of *E. coli acrA*, *acrB*, or *tolC* were used. pSpec elicits resistance to Str and Spec using an encoded protein, AadA—streptomycin 3' adenylyltransferase, that prevents the binding of Str or Spec to the 30S ribosomal subunit thus preventing protein synthesis (163, 164). pKan elicits resistance to Kan when conjugated into the cell by way of the encoded AphA protein, an aminoglycoside transferase. AphA transfers phosphate to the hydroxyl group of kanamycin therefore preventing the conventional activity of kanamycin, which is to bind to 30S subunit to prevent ensuing protein synthesis (165, 166). Primers were used to isolate the following genes from the *E. coli* genome using PCR amplification: *tolC, acrB,* and *acrA* (Table 4.1). Each primer was designed with restriction enzyme cleavage sites at the beginning and end of the amplified nucleotide sequence that matches to corresponding cleavage sites already present in the multiple cloning region of the pKan and pSpec expression vectors. The primers used to amplify *tolC* are labelled: 5'tolcF and 3'tolcR. The primers used to amplify *acrA* are labelled: 5' AcrA F and 3' AcrA R. The primers used to amplify *acrB* are labelled: 5' AcrB F and 3' AcrB R. The *E. coli* genes were isolated from a whole genome preparation (Wizard

Genomic Preparation Kit, Promega) of *E. coli* using iProof™ High-Fidelity PCR Kit (Bio-Rad). *E. coli* gene *acrB* and pSpec were digested with respective restriction enzymes that then allowed the directional ligation of *acrB* into the now cleaved nucleotide sequence of the pSpec vector to create p*acrB*, while *acrA* went through the same procedure with and be ligated into pKan to create p*acrA*. 1μL of T4 DNA Ligase and 1 μL DNA ligase buffer (NEB) were used in a 10 μL reaction for ligation to proceed. *tolC* was placed in both pSpec to created p*tolC*. Then each newly created vector was introduced into electrocompetent *E. coli* Top 10 cells (Life Technologies) using a 2.5 kV pulse of electricity (Gene Pulser Xcell, Bio-Rad, Hercules, CA). The *E. coli* Top 10 cells containing the vector were grown on a plate containing LB agar media with the corresponding antibiotic for selection of the presence of the vector in the *E. coli* Top 10 cells overnight at 37°C. Colonies present on the plate the next day were confirmed for the presence of the gene using colony PCR in 30μL reaction of DNA polymerase GoTaq® (Promega). The primers used in the reaction mix to identify the presence of the *E. coli* gene in the correct location are either "568F" and "568R" or "687F" and "687R" (Table 4.1). The 568F/R set of primers mark sites for amplification on the nucleotide sequence from either side of the multiple cloning region of pSpec, while the 687F/R represent nucleotide regions to amplify from either side of the multiple cloning region of pKan. If the *E. coli* gene was absent from the region then a 500bp DNA fragment was present when the DNA obtained from the PCR reaction is run through DNA gel electrophoresis. If the *E. coli* gene was present in the plasmid, the size of the gene plus 500bp was the size of the amplified DNA present on the DNA agarose gel (Table 4.1). The nucleotide sequence of the plasmid was also obtained for final confirmation. Any colonies

confirmed to have the vector of interest were grown again overnight to allow for isolation of the plasmid using a Miniprep Plasmid Purification Kit (Qiagen). The resulting plasmid descriptions are summarized in Table 4.2. Then the vector was isolated and it, along with pRL528, a helper plasmid that has methylases AvaIIM and Eco47IIM to prevent restriction enzyme activity of *Synechocystis* cells (161, 167), was electroporated into electrocompetent Top 10 *E. coli* cells. The Top 10 *E. coli* cells with the vectors were plated on LB media with antibiotics corresponding to the resistance conferred by the two vectors within the cell. A colony PCR using GoTaq DNA polymerase reaction mixture (Promega) was used to confirm the presence of the expression vector containing the expected *E. coli* gene.

4.2.3 Conjugation of cyanobacteria to create strains

Table 4.3 Strains used to Add *E. coli* genes to SD100 and SD277

Strain Name	Description	Parent	Curtiss Collec.	Reference
SD100 p*acrB*	SD100 p⋃691 (Ec *acrB)*	SD100	SD643	This research
SD277 p*acrB*	SD277 p⋃691 (Ec *acrB)*	SD277	SD644	This research
SD100 p*acrA*	SD100 p⋃692 (Ec *acrA)*	SD100	SD645	This research
SD277 p*acrA*	SD277 p⋃692 (Ec *acrA)*	SD277	SD646	This research
SD100 p*tolC*	SD100 p⋃699 (Ec *tolC)*	SD100	SD647	This research
SD277 p*tolC*	SD277 p⋃699 (Ec *tolC)*	SD277	SD648	This research
SD100 pacrA p*tolC*	SD100 p⋃692 (Ec *acrA)* p⋃699 (Ec *tolC)*	SD100	SD649	This research
SD277 pacrA p*tolC*	SD277 p⋃692 (Ec *acrA)* p⋃699 (Ec *tolC)*	SD277	SD650	This research
SD100 pacrA p*acrB*	SD100 p⋃691 (Ec *acrB)* p⋃692 (Ec *acrA)*	SD100	SD651	This research
SD277 pacrA p*acrB*	SD277 p⋃691 (Ec *acrB)* p⋃692 (Ec *acrA)*	SD277	SD652	This research

In order to get the expression vectors varying in size from 7.2kb to nearly 10kb in nucleotide lengths into SD277 or SD100, conjugation must be performed to alleviate the effects of native restriction enzyme digestion in the cyanobacterial cells using an

established method (161, 167). To begin the combination, two groups of *E. coli* cells were grown overnight at 37ºC in 15mL liquid LB cultures overnight bearing the following vector or sets of vectors: one set of *E. coli* the expression vector with pRL528 and conjugal plasmid pRL443 that facilitates the transfer of plasmids between cells. pRL443 is an RP4 derivative plasmid that is essential for conjugation of the *E. coli* gene containing plasmid into SD100 or SD277. RP4 (and its derivatives) have two gene regions that encode the function of the plasmid: Tra1 and Tra2 (168). Tra1 is composed of three operons that encode the primase, relaxase, and leader operon (169). Tra2 is composed of 11 operons that are responsible for mobilizing the RSF1010 vector (or vector based on RSF1010); RSF1010 is not self-transmissible into a cell (168). Simultaneously, 10mL of the cyanobacteria was grown in liquid BG-11 culture to an OD_{730} of 0.4. Then all three sets of cells underwent a washing process in which they were centrifuged at 3000 x g for 10 min. The supernatant was poured off and the bacterial pellet was resuspended in 10mL of new BG-11 media. This process was repeated two more times. After the final wash, the cells were resuspended in 5mL of BG-11. Then a control group was created in which 1mL of cyanobacteria was added to 0.5mL of *E. coli* Top cells containing the expression vector with pRL528. Simultaneously, a conjugation group was created in which 1mL of cyanobacteria are added to 2mL of *E. coli* containing pRL443 and 2mL of the *E. coli* cells containing both the expression vector and pRL528. These cultures were mixed and then sit for 20 min. Then 400 µL of each of the two experimental cell mixtures was spread onto a nitrocellulose membrane that had been placed on BG-11+0.5% LB media. This then sat on the lab bench overnight. The next morning it was put in a SANYO illuminated chamber at 30ºC with light until the plate

had a light green lawn (usually after a day or two). Then the nitrocellulose membrane was moved to BG-11 with antibiotics corresponding to the resistances specified by the expression vector(s) in an effort to select for cyanobacteria colonies containing that vector. The antibiotics added to the BG-11 media, kanamycin sulfate, streptomycin sulfate, and spectinomcin dihydrochloride pentahydrate, were purchased from Sigma Aldrich. Bright green colonies arrived on the nitrocellulose membrane within a week for these successfully modified cells. The DNA modifications were confirmed using colony PCR using the same primers that were originally used to amplify the nucleotide sequence of the *E. coli* gene from the *E. coli* chromosome (Table 4.1), in the method described in Chapter 3.2.2, to ensure its existence in the cyanobacteria. Subsequently, the cyanobacterial cells were grown to the optical density and volume described in Chapter 4.2.1 to test for the existence of mRNA corresponding to each of the *E. coli* genes using reverse transcriptase-PCR (RT-PCR). I created 14 strains for this chapter including the ones with the exogenous genes from *E. coli* and those containing empty vectors, representing controls to ensure the results observed are not solely caused by the addition of the vector. The properties of all of these strains are summarized in Table 4.3.

4.2.4. RT-PCR to identify gene expression

A 40mL culture was grown to mid-log level phase as explained previously, which was then centrifuged at 6000 x g for 10 min to pellet the cells. The supernatant was removed and 500 μL of BG-11 media and 1 mL of RNAprotect Cell Reagent (Qiagen) was added. Each sample was stored at -20^{o}C until it was ready for RNA isolation. For mRNA extraction, the Qiagen RNeasy Kit was used. Briefly, the stored samples were thawed and pelleted using a centrifuge at 6000 x g for 10 min. The supernatant was removed, and the

pellet was resuspended in 700 μL of RLT Buffer (Qiagen) and transferred to a 2 mL locking lid microcentrifuge tubes (Eppendorf, Hamburg, Germany) containing 0.2 g of 0.25 mm-diameter acid washed glass beads (Sartorius Stedim, Göttingen, Germany). The mixture was placed in a Bullet Blender Storm 24 homogenizer (Next Advance, Troy, NY) and homogenized for 5 min at the maximum speed. The remaining extraction steps were followed exactly as Qiagen recommended. mRNA was quantified using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Each mRNA sample (1 μg) was treated with one unit of RQ1 RNase-free DNase (Promega) according to the manufacturer's instructions. To create the cDNA, 250ng of RNA was transcribed using the Qiagen One-Step RT-PCR Kit in a final reaction volume of 50 μL and then placed in the thermal cycler using the manufacturer's recommendations, using primers previously published to identify *E. coli* genes *acrA*, *acrB*, *tolC* (64) and separately a set of primers from previously published work was used to identify *petB*, a constitutively expressed *Synechocystis* sp. PCC 6803 gene (170), ensuring that mRNA was isolated from all cyanobacterial samples. The primers used for RT-PCR analysis are summarized in Table 4.4.

Table 4.4 Primers used for RT-PCR analysis of *E. coli* genes added to SD100 and SD277

Primers for RT-PCR	Sequence	Reference
petB-F	CCTTCGCCTCTGTCCAATAC	(170)
petB-R	TAGCATTACACCCACAACCC	(170)
acrA-F	CTCTCAGGCAGCTTAGCCCTAA	(64)
acrA-R	TGCAGAGGTTCAGTTTTGACTGTT	(64)
acrB-F	GGTCGATTCCGTTCTCCGTTA	(64)
acrB-R	CTACCTGGAAGTAAACGTCATTGGT	(64)
tolC-F	AAGCCGAAAAACGCAACCT	(64)
tolC-R	CAGAGTCGGTAAGTGACCATC	(64)

4.2.5 Tolerance Assays to determine half maximal inhibitory concentrations (IC_{50})

The following process is used in Chapter 3. But briefly, each of the strains was grown to an OD_{730} (0.7-0.8) representing a mid-log level growth phase in 10 mL culture under aeration, illumination, and at 30°C. The cells were then diluted to an OD_{790} of 0.40 and incubated in 96-well plates (Corning) for 3 days in the illuminated incubator (SANYO), with each well housing a specific, yet different concentration of each of the antibiotics or R6G. The final OD_{790} was measured using a plate reader (Biotek). The results were submitted for analysis using software to determine the non-linear variable slope curve that best fits the regression of the concentration of cyanobacteria, indicating their tolerance to the antibiotic.

4.2.6 FFA separation and gas chromatography measurement

The processing of each of the strains presented in this chapter follows the exact procedure from Chapter 3.2.5 to achieve samples representing intracellular FFA concentrations and distinct samples representing extracellular FFA concentration after each strain is grown to a final density of $8x10^8$ to $1.1x10^9$ cells/mL. The samples were processed using a GC using nitrogen as the carrier gas. The integral of peaks at specific time points were then analyzed by being compared to a series of standards that identify the time that each peak arrives based on the temperature of the oven in the GC and the amount of each sample carrier based on the integral of the peak.

4.3 Results and Discussion

4.3.1 Tolerance to toxic chemicals is modified when genes encoding components of the AcrAB-TolC efflux pump are added to SD100 and SD277

Table 4.5 IC_{50} of Addition Strains to Ampicillin, Chloramphenicol, Erythromycin, and Rhodamine 6G in which IC_{50} of derivative strain is significantly different than SD100 unless otherwise indicated with ns for not significant.

	SD100	SD100 p*acrA*	SD100 pKan	SD100 p*acrB*	SD100 p*tolC*	SD100 pSpec	SD100 p*acrA* p*tolC*	SD100 p*acrA* p*acrB*	SD100 pSpec pKan
Treatment	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL
Amp	955.2 ± 256	1315 ± 77 (ns)	1151 ± 82 (ns)	1109 ± 123 (ns)	1038 ± 119 (ns)	658.6 ± 88.3	1705 ± 154	914.9 ± 52.9 (ns)	516 ± 78
R6G	6.08 ± .871	4.51 ± 0.7 (ns)	5.1 ± 0.6 (ns)	5.44 ± 0.34 (ns)	6.7 ± 0.8 (ns)	3.44 ± 0.58	7.8 ± 0.9 (ns)	7.38 ± 1.06 (ns)	6.35 ± 0.31 (ns)
Cm	2.290 ± 0.401	1.444 ± 0.114	1.390 ± 0.186	1.976 ± 0.734 (ns)	1.93 ± 0.257 (ns)	1.653 ± 0.205 (ns)	1.781 ± 0.303 (ns)	2.004 ± 1.199 (ns)	2.571 ± 0.446 (ns)
Ery	0.202 ± 0.02	0.080 ± 0.003	0.072 ± 0.007	0.138 ± 0.080 (ns)	0.119 ± 0.026 (ns)	0.079 ± 0.005	0.246 ± 0.009	0.334 ± 0.038	0.122 ± 0.086 (ns)

Significant increases in tolerance are highlighted in green

Table 4.6 IC_{50} of Addition Strains to Ampicillin, Chloramphenicol, Erythromycin, and Rhodamine 6G in which IC_{50} of derivative strain is significantly different than SD277 unless otherwise indicated with ns for not significant.

	SD277	SD277 p*acrA*	SD277 pKan	SD277 p*acrB*	SD277 p*tolC*	SD277 pSpec	SD277 p*acrA* p*tolC*	SD277 p*acrA* p*acrB*	SD277 pSpec pKan
Treatment	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL
Amp	1570 ± 170	1117 ± 21	76.67 ± 28.5	118.2 ± 8.7	1577 ± 197 (ns)	412.6 ± 34.8	1615 ± 35 (ns)	1126 ± 88	1348 ± 217 (ns)
R6G	1.41 ± 0.12	3.61 ± 0.42	4.91 ± 0.87	2.95 ± 0.13	4.12 ± 0.96	4.06 ± 0.28	2.97 ± 0.17	5.44 ± 3.06	4.28 ± 0.34
Cm	4.749 ± 1.043	1.833 ± 0.390	1.793 ± 0.430	1.984 ± 0.527	2.951 ± 0.23	2.96 ± 0.4	2.616 ± 0.525	6.637 ± 0.969 (ns)	1.596 ± 0.238
Ery	0.135 ± 0.03	0.091 ± 0.034	0.149 ± 0.016	0.080 ± 0.008	0.189 ± 0.041 (ns)	0.055 ± 0.004 (ns)	0.253 ± 0.022	0.166 ± 0.021	0.136 ± 0.011

Significant increases in tolerance are highlighted in green.

In Table 4.5 and Table 4.6, I have highlighted significant increases of tolerance to chemicals in green when the derivative strain is compared to the parent. The most notable picture presented is that there are not many strains possessing increased tolerance to Amp, Cm, Ery, or R6G in Table 4.5, representing SD100 and its *E. coli* gene addition derivatives and control strains. The most consistent SD100-based strain that had increased tolerance to Amp, Cm, Ery, and R6G was SD100 p*acrA* p*tolC*, which had greater tolerance to Amp and Ery and no significant difference of tolerance to R6G and Cm relative to SD100. The only other SD100 derivative that has increased tolerance relative to SD100 was SD100 p*acrA* p*acrB* when treated with increasing concentration of Ery (Table 4.5). There is no clear pattern that any addition of individual or two of the *E. coli* genes that encode the AcrAB-TolC efflux pump increase the tolerance of SD100 to Amp, R6G, Cm, or Ery.

This lack of increased tolerance to these toxic chemicals is parallelly depicted in the SD277 derivative strains with *E. coli* gene additions or the control vectors (Table 4.6); however, every derivative strain of SD277 featured in this chapter has an increased tolerance to R6G relative to SD277. This increase cannot be attributed to the added *E. coli* genes because the control vector addition provides the same outcome. The only two strains that were derivatives of SD277 that possessed increased tolerance to Ery relative to SD277 were SD277 p*acrA* p*tolC* and SD277 pKan (Table 4.6). Again, this increase cannot be attributed to the presence of the *E. coli* genes in SD277 because pKan caused a similar significant increase. This increase in tolerance by the addition of pKan or pSpec is not seen in the SD100 derivatives. All SD100-based strains containing a control plasmid either do not change or have a decreased tolerance to Amp, Cm, Ery, and R6G relative to

SD100 (Table 4.6). To address my concern that the *E. coli* genes are not being expressed in SD100 or SD277, I performed RT-PCR analysis of each strain that contained an exogenous gene while using SD100 and SD277 as controls (Figure 4.1). Every strain possessed the appropriate mRNA. SD100 and SD277 did not contain *tolC*, *acrA*, or *acrB* mRNA, but did possess mRNA encoded by the native gene *petB* (Figure 4.1). All of the *E. coli* gene addition strains featured in this chapter also possessed mRNA encoded by *petB*. So, the *E. coli* genes are expressed, yet there exists no tolerance increase to toxic chemicals, but the experiment did show that the control vectors modified tolerance. In addition, there are no differences in growth rates among any of the derivative strains relative to the respective SD100 or SD277 parent strain (Figure 4.6 and Figure 4.7).

I did not think an expression vector added to a cyanobacteria cell could change the tolerance to toxic chemicals, but some research exists indicating that the presence of exogenous plasmids can modify resistance to antibiotics. This is not a new phenomenon in the world of genetically modifying bacteria. But the basics follow conventional logic. Plasmids require a significant amount of energy to maintain in the cell between the replication of the DNA and the resulting protein synthesis. This was shown in 1987, in which four different pUC8 plasmids were added to *E. coli*, varying in size from 1.7kb to 6kb in which researchers noticed that even though the plasmids conferred resistance to Amp, that the larger the plasmid, the lower the tolerance to Amp (171). When the RSF1050 plasmid was added to *E. coli*, a relative plasmid to RSF1010—the basis for pKan and pSpec (172), the expression rates of the genes located in the plasmids decreased as the plasmid copy number increased (173). These pUC8 plasmids did possess up to 300 copies in a single *E. coli* cell (174). The plasmid copy number of pKan and

pSpec is likely a single copy per chromosome, which amounts to 10-14 per cell in SD100 and SD277. We have performed unpublished estimates of copy number in our lab based on the quantity of DNA from exogenous plasmids compared to the DNA of the chromosome using a colony PCR of the same cyanobacterial sample but with different primers, amplifying the same length nucleotide sequence from either the plasmid or the chromosome. While this is not as accurate as other methods with actually isolating the plasmids from the cells, we certainly learned there is not 300-times the amount of plasmid DNA or even 10-times the amount in a cyanobacterial cell.

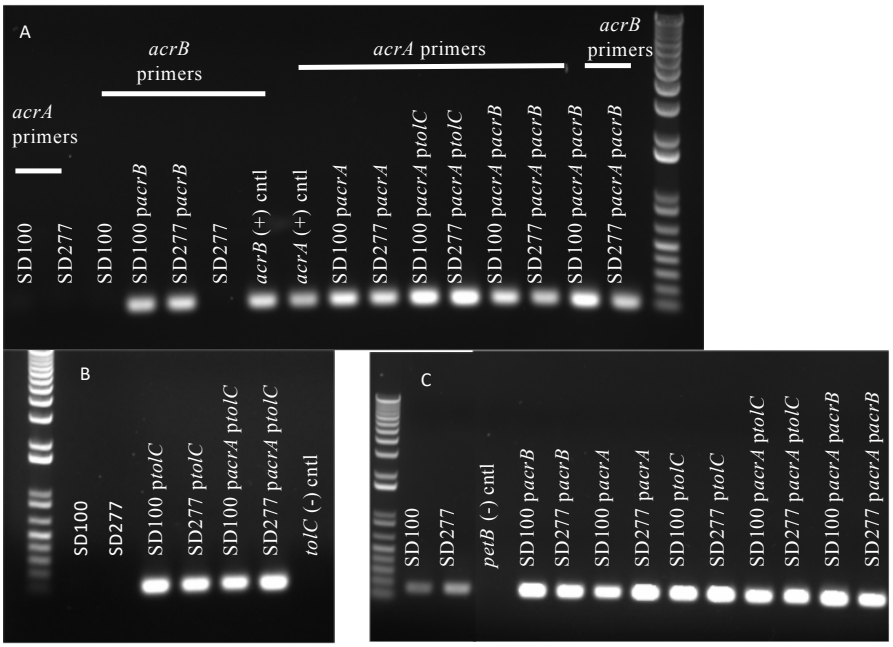


Figure 4.1 DNA Gel Electrophoresis displaying DNA Fragments from RT-PCR reactions of each the *E. coli* gene in the complementation strains (A) *acrA* and *acrB,* (B) *tolC* and (C) *petB*

4.3.2 Addition of genes encoding multiple components of the AcrAB-TolC efflux pump modifies extracellular and intracellular FFA concentrations

Figure 4.2 Extracellular Free Fatty Acid Concentrations of the SD277-Based Addition Strains where * is p < 0.05, ** is p < 0.005, *** is p < 0.0005, **** is p < 0.0001, and ns is not significant

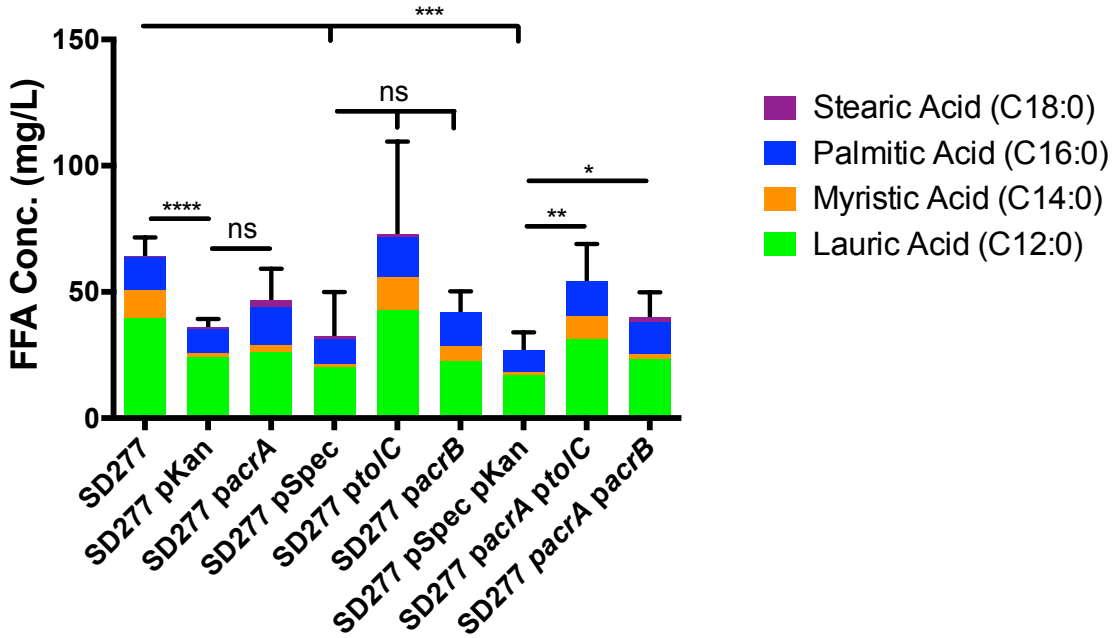


Figure 4.3 Intracellular Free Fatty Acid Concentrations of the SD277-Based Addition Strains where * is p < 0.05, ** is p < 0.005, *** is p < 0.0005, **** is p < 0.0001, and ns is not significant

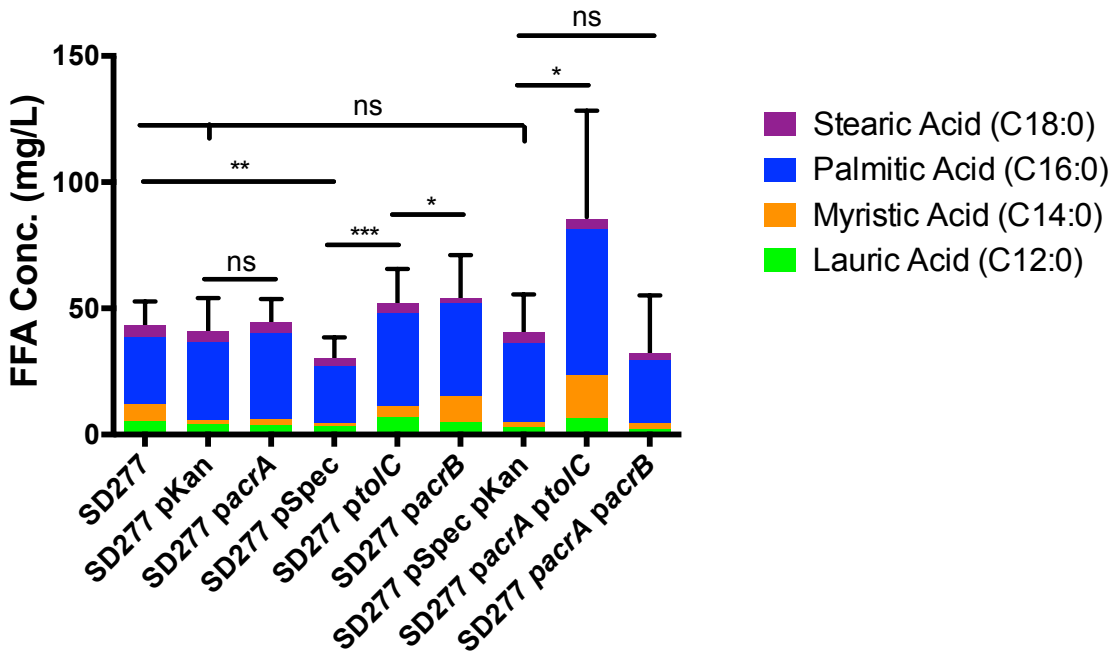


Figure 4.4 Extracellular Free Fatty Acid Concentrations of the SD100-Based Addition Strains where * is p < 0.05, ** is p < 0.005, *** is p < 0.0005, **** is p < 0.0001, and ns is not significant

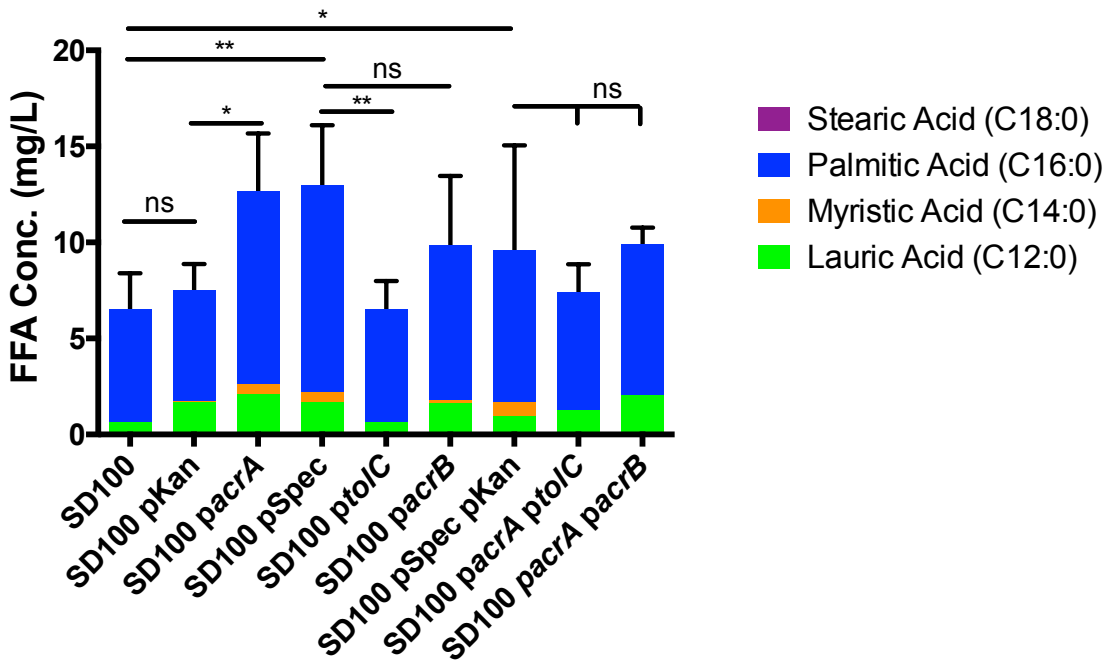
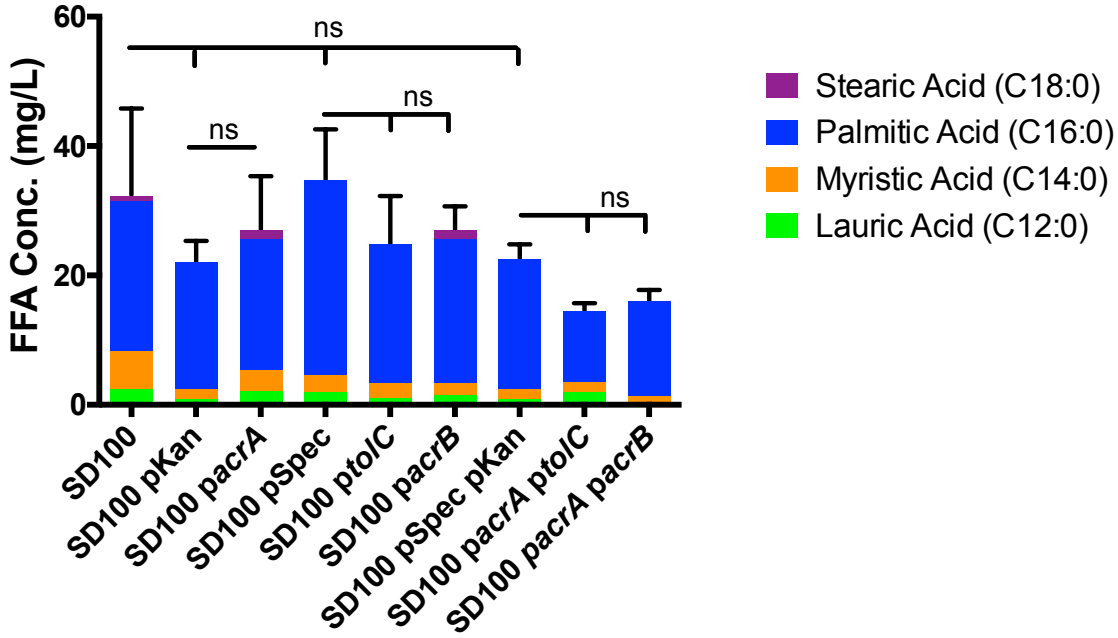


Figure 4.5 Intracellular Free Fatty Acid Concentrations of the SD100-Based Addition Strains where ns is not significant



To achieve maximal FFA concentrations, the cyanobacteria cells are grown to late-log phase as bacteria do not start to accumulate FFAs in optimal quantities until they reach that growth phase. With the addition of these *E. coli* genes, *acrA*, *acrB*, and *tolc*, the goal was to secrete more FFAs than the parent strain. None of the SD277-based strains had a higher concentration of extracellular FFAs relative to SD277 (Figure 4.2). Neither SD277 p*acrA* p*tolC* nor SD277 p*acrA* p*acrB* achieved a greater extracellular FFA concentration compared to its respective control strain SD277 pKan pSpec (Figure 4.2). However, SD277 p*acrA* did not have a modified extracellular FFA concentration relative to SD277 pKan, its control strain (Figure 4.2). This lack of difference in extracellular FFA concentrations was also seen in SD277 p*tolC* and SD277 p*acrB* relative to SD277 pSpec.

None of the SD277 derivative strains tested had a significantly lower intracellular FFA concentration relative to SD277 with the exception of SD277 pSpec (Figure 4.3). But both SD277 p*tolC* and SD277 p*acrB*, which both contain pSpec which is the parent plasmid for the *tolC* and *acrB* additions, possessed significantly increased intracellular FFA concentrations relative to SD277 pSpec (Figure 4.3). The intracellular FFA concentration of SD277 p*acrA* p*tolC* is greater than SD277 pKan pSpec, while SD277 p*acrA* p*acrB* is the same as SD277 pKan pSpec. SD277 pKan pSpec has a significantly increased FFA concentration intracellularly when compared to SD277 (Figure 4.3). However, neither SD277 pKan nor SD277 p*acrA* have an altered intracellular concentration relative to SD277 (Figure 4.3). None of the strains derived from SD277 varied in growth rates when compared to SD277 (Figure 4.6). Interestingly, SD277 p*acrA*

p*tolC*, SD277 p*acrA* p*acrB*, SD277 p*tolC*, and SD277 p*acrA* had a visible oil sheen when grown to late log phase that was not observed in SD277 (Appendix A.1)

The results obtained from SD100 and its derivatives are far simpler to explain. SD100 pSpec and SD100 pSpec pKan had significantly increased extracellular FFA concentrations relative to SD100, while SD100 pKan was not different than SD100 (Figure 4.4). SD100 p*acrA* had a significantly greater extracellular FFA concentration than SD100 pKan, but SD100 p*tolC* was significantly less than SD100 pSpec (Figure 4.4). SD100 p*acrB* was unchanged relative to SD100 pSpec with respect to extracellular FFA concentrations. Neither SD100 p*acrA* p*tolC* nor SD100 p*acrA* p*acrB* had significantly different extracellular FFA concentrations relative to SD100 pSpec pKan (Figure 4.4). None of the intracellular FFA concentrations varied significantly when compared to SD100 (Figure 4.5).

As in Chapter 3, I find it difficult to draw any conclusion from the SD100 derivative strains because the intracellular FFA concentrations were not modified so the plasmids are not affecting native FFA synthesis in SD100. Importantly, the addition of the plasmids does not affect the growth rates (Figure 4.7). In addition, the extracellular FFA concentrations are either increased or not changed in every derivative strain relative to SD100, but I do not see a clear pattern. Some, like SD100 p*acrA*, increase extracellular FFA concentration dramatically compared to SD100 while SD100 p*tolC* is not different (Figure 4.4). The aforementioned results were triplicated so the data is readily reproducible. Also, SD100 pSpec has a significantly greater concentration of extracellular FFAs, so none of the extracellular FFA concentration increases can be directly attributed to the presence of *E. coli* genes. But I would note that the addition of the plasmids does

not hamper FFA efflux in SD100 cells, which is not the case for the SD277 derivative strains. The extra FFA synthesis forced in SD277 cells likely has an impact on this result.

Furthermore, looking at the results of the intracellular FFA concentrations of three control strains derived from SD277, the SD277 pSpec strain possessed a significant decrease in intracellular FFA production relative to SD277, while SD277 pKan and SD277 pKan pSpec were unchanged. However, all three control strains possessed a significant decrease in extracellular FFA concentration relative to SD277. But the most exciting new to come out of this chapter is that SD277 p*acrA* p*tolC* and SD277 p*acrA* p*acrB* have a significant increase in extracellular FFAs relative to SD277 pKan pSpec, which indicates that the *E. coli* transport genes are encoding proteins that function in *Synechocystis* sp. PCC 6803. If these proteins did not have a role in these SD277 derivative strains, then I would not expect this increase in extracellular FFA concentration. Combining this FFA export evidence with previously established sugar transport in cyanobacteria using exogenous proteins leads to a broad picture of all of the potential uses of exogenous transport system to move chemicals produced within cyanobacteria from the cytoplasm to the extracellular environment.

But, this decrease of extracellular FFA concentration of the SD277 control strains could be caused by either decreased transportation rate of the FFAs from within the cell to the extracellular environment or from the decreased production rate. I suspect this decrease is caused by a decreased production rate of FFA therefore limiting the amount of substrate that the transport proteins, both native and exogenous, have to use. If the transportation rate has been changed through the addition of these plasmids, then I would expect to see an increase in intracellular FFA concentration just as observed with the

sll0180 or *slr2131* mutation strains derived from SD277. But since intracellular is either not changed or decreased in the three control strains, the evidence suggests that the production of FFA is hampered because of the addition of exogenous plasmids. Additionally, there is some previous research findings that corroborate this evidence.

In numerous bacteria including *Bacillus subtilis* and *Vibrio cholerae*, the inducible SOS DNA repair system is modulated, in part, by LexA that functions as a repressor. Once DNA damage occurs, ssDNA is formed which binds to RecA. This combination cleaves the LexA dimer that function as a repressor to the SOS system, initiating DNA repair (175). The protein encoded by a *lexA* homolog in *Synechocystis* sp. PCC 6803, Sll1626, was shown to have a role in down-regulating *fab* (fatty acid biosynthesis) genes in a genetically modified version of *Synechocystis* sp. PCC 6803 using some of the same modifications present in SD277 to promote FFA synthesis (176). LexA in *V. cholerae* has been shown to induce the SOS DNA repair response when plasmids are conjugated into the cell. The LexA homolog found in *Synechocystis* sp. PCC 6803, unlike that in *E. coli* and *V. cholerae*, is not linked to any genes involved in DNA metabolism in *Synechocystis* sp. PCC 6803 (177–179). So *lexA* may not be involved in SOS DNA repair in *Synechocystis* sp. PCC 6803; however, LexA still may be induced by exogenous plasmids and has been shown to act directly on genes controlling fatty acid biosynthesis in SD277 or *Synechocystis* sp. PCC 6803. A future study removing *lexA* from the strains created for this research may help illustrate a picture of involvement not only, in fatty acid biosynthesis but may help explain the decrease FFA concentrations in SD277 control derivative strains containing exogenous plasmids lacking the *E. coli* transport genes. Importantly, my research has linked the addition of exogenous plasmids

to a decrease in production of FFAs in SD277. In addition, now that this research has established efficacy of *E. coli acrA* and *acrB* genes in the cyanobacterial cell, placing the entire *E. coli* multidrug efflux system in the cells simultaneously (AcrAB-TolC) may increase FFA concentration extracellularly.

The nucleotide sequence identity of *sll0180* to *E. coli acrA* is not as great as the identity of *slr2131* and *slr1270* is to *E. coli acrB* and *tolC*, respectively. *sll0180* is as close a homolog that the cyanobacteria possess, yet the open reading frame is about 40% longer than that of *acrA*, while *slr1270* and *slr2131* share far more similarities to their homologous *acrB* and *tolC*, respectively. Each have genes that are far closer in size and sequence, with resulting proteins, in the case of Slr1270, that fold and function in the same manner as TolC in *E. coli* (60). Each of these *E. coli* genes used in this chapter were placed under the control of a Ptrc promoter that is not inducible or repressible. Having an inducible promoter may benefit the total FFA efflux. In *Salmonella enterica*, a closely related bacteria to *E. coli*, the expression of *acrAB* is subject to active regulation by OmpR that binds to promoter sites of each gene (180). While growth rates of the strains do not change (Figure 4.6 and Figure 4.7), the synthesis of the efflux proteins may need to be modulated for optimal efficacy.

Other researchers have used transporters to successfully secrete FFA. In a high FFA producing *E. coli* strain, researchers were able to secrete 110 mg/L of FFA into the extracellular environment using a modified expression level of *E. coli* MsbA transporter mRNA (181). Until this study, no reports had existed of MsbA being used in FFA transport. This increase was 20 times over the control strain that was performed by using 4 genetic modifications to promote FFA production. These FFA producing modifications

included the addition of *tesA* and *AtFatA*, both of which are thioesterases as described previously in Chapter 3. Also, *fadD* and *fadL* were removed as the proteins synthesized from the genes facilitate uptake of long chain FFA, which would reverse the secretion. It's surprising that the secretion of a few of the strains created in this research is comparable to that of a genetically modified *E. coli*. The maximum secretion of total FFAs achieved with a SD277-based strain in this chapter was between 40 and 42 mg/L, about half of the concentration shown in *E. coli*. It should be considered that other *E. coli* strains have been shown to make in the range of 8.6 g/L of FFA through various genetic engineering modifications (182), compared to the 197 mg/L observed in SD277 when the entire cell and supernatant is processed for FFA concentration (14). But to increase the efficiency of biofuel productions, the cells need to be growing continuously. While the 8.6 g/L measured in a genetically modified *E. coli* strain is a far greater concentration of FFA when compared to SD277, the FFAs are only accessible after the FFAs have been extracted from the *E. coli*. When comparing extracellular FFA concentrations, the maximal achieved concentration when comparing SD277 to a genetically modified *E. coli* strain is in the same log level, so my research in this dissertation is an important step to understanding how to efficiently move FFA out of the cell.

In a similar study, trying to retrieve secreted FFA from a continuously growing *E. coli* strain that was engineered to have the genes encoding the acyl-ACP from *Umbellularia californica* (*fadD*, *fadE*, and *fadAB*) placed in the chromosome repeatedly, which would allow the encoding of a protein that would compete with FFA synthesis. The goal was to produce an *E. coli* with a high FFA yield with an antibiotic-resistance carrying plasmid present. They achieved slightly more than 200 mg/L of lauric acid and

palmitic acid in the collected foam that forms from the growth of the bacteria (183). Again, this is in the same log level concentration of the FFAs observed in SD277. So, cyanobacteria are not at an immense disadvantage is extracellular FFA concentrations, but instead cyanobacteria are nearly identical to observed *E. coli* levels. Combine this information with the idea that cyanobacteria can be grown outside with limited nutrients added to the growth medium and the industrial relevance of these modified cyanobacteria is evident.

Figure 4.6 Growth Curve of SD100-Based Deletion Strains

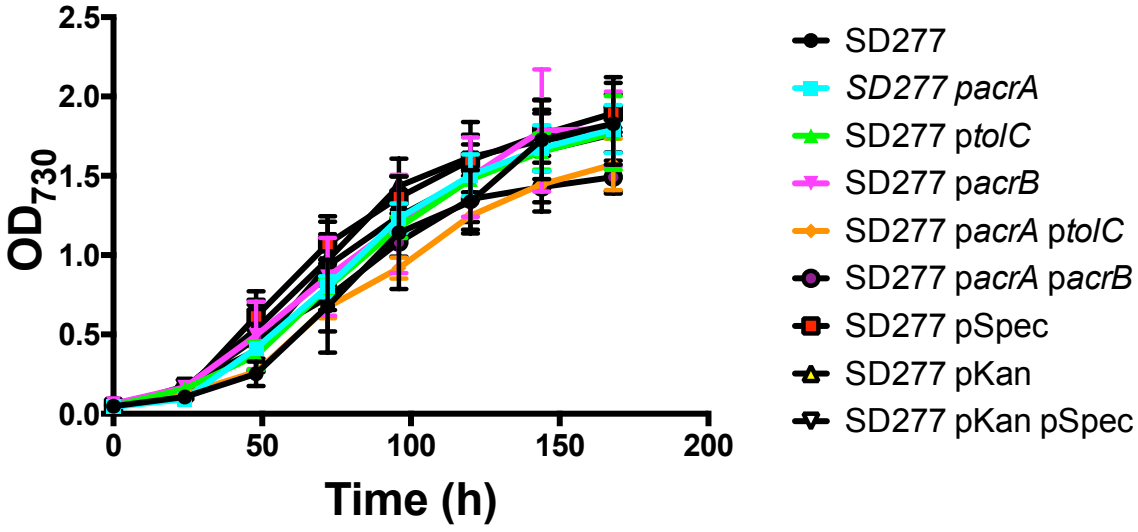
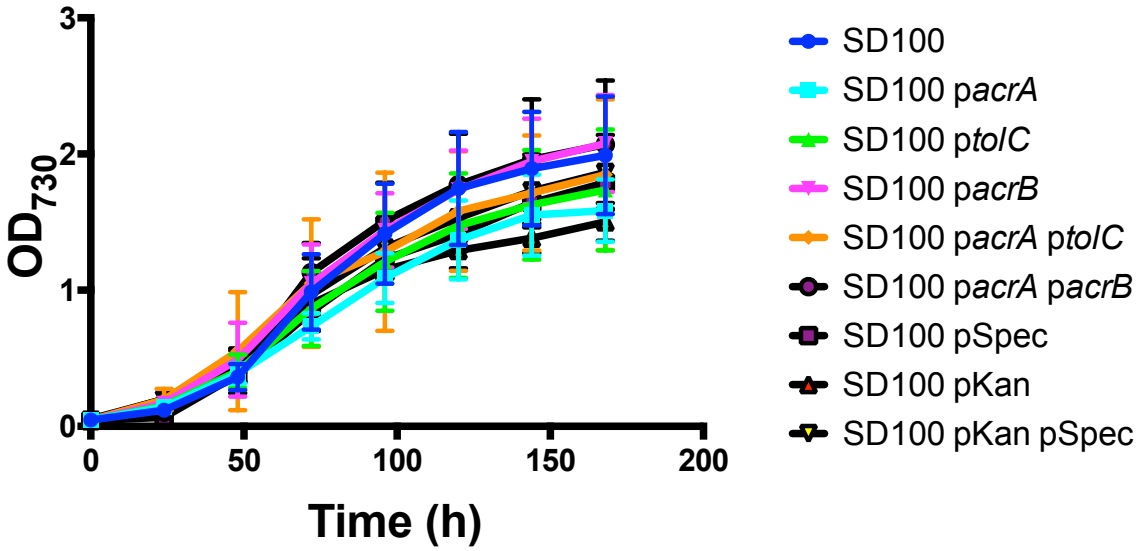


Figure 4.7 Growth Curve of SD100-Based Deletion Strains



4.4 Conclusion

Tolerance to various antibiotics was not increased universally. Only SD100 p*acrA* p*tolC* possessed a significant increased tolerance to Amp, while SD100 p*acrA* p*acrB* and SD100 p*acrA* p*tolC* had a significant increase of tolerance to Ery. All SD277-based strains possessed an increased tolerance to R6G, but it was not due solely to the addition of the *E. coli* genes as all of the plasmid vectors had a significantly increased tolerance to R6G. SD277 pKan and SD277 p*acrA* p*tolC* both had increased tolerances to Ery, but this again only indicates that the tolerance is not due to the addition of the *E. coli* genes in the SD277-based strains.

Some informative results were delivered when the concentration of extracellular FFAs were measured. Both SD277 p*acrA* p*tolC* and SD277 p*acrA* p*acrB* possessed a significant increase in extracellular FFA concentrations relative to SD277 pSpec pKan, indicating that all three *E. coli* transport proteins function in *Synechocystis* sp. PCC 6803. SD277 *tolC* and SD277 *acrB* possessed statistically the equivalent extracellular FFA concentrations compared to SD277 pSpec, while SD277 *acrA* and its vector control possessed the same pattern. The intracellular FFA measured in this chapter were either statistically identical to SD277 or increased with one exception: SD277 pSpec. These results presented the possibility that the plasmid may be affecting FFA production itself, which may be governed by a LexA homolog in SD100 (and SD277).

The most important piece of information garnered from this chapter is that these *E. coli* efflux genes can be expressed in SD100 and SD277, compensating for the negative effects that the addition of the plasmids creates with respect to FFA production and tolerance to toxic chemicals. This information can be used to further the study of using exogenous transporter in SD277 including in Chapter 5 of this dissertation.

CHAPTER 5

COMPLEMENTATION OF THE REMOVED *SYNECHOCYSTIS* SP. PCC 6803 TRANSPORT GENES WITH HOMOLOGOUS *ESCERICHIA COLI* GENES FURTHER MODIFIES THE FREE FATTY SECRETION AND TOLERANCE TO ANTIBIOTICS AND A DYE

5.1 Introduction

5.1.1 Outline and hypotheses

In Chapter 3, some lethal effects of the deletions of *slr2131* and *sll0180* from SD100 and SD277 were observed when the mutant strains were grown in the presence of antibiotics and R6G. This chapter seeks to address this by adding the homologous *E. coli* gene to complement the mutations, using the plasmids developed in Chapter 4. In many studies examining the effects of the removal of RND efflux pumps, researchers remove the genes of interest and then add them back to the bacteria using an expression vector. I use this same complementation strategy in this chapter. The removal of *slr2131* and *sll0180* from SD277 decreased the concentrations of extracellular FFA and increased the intracellular FFA concentrations. Subsequently, the opposite occurred by the removal of *slr2131* and *sll0180* from SD100 in which the extracellular FFA concentrations increased, but intracellular concentrations were unchanged. The tolerance to toxic chemicals mostly decreased in the mutant strains when compared to the respective parent strains.

I hypothesize that the addition of p*acrB* to SD100 Δ*slr2131* and SD277 Δ*slr2131* and the addition of pS*acrA* to SD100 Δ*sll0180* and SD277 Δ*sll0180* will restore the decreased IC_{50} that occurred when the original deletion mutants were treated with various

concentrations of Amp, R6G, Cm, and Ery as observed in Chapter 3. In addition, I hypothesize that the addition of p*acrB* to SD277 Δ*slr2131* and pS*acrA* to SD277 Δ*sll0180* will significantly increase extracellular FFA concentrations and decrease intracellular FFA concentrations compared to their respective deletion mutant parent strains. I also think that the addition of p*acrB* to SD100 Δ*slr2131* and pS*acrA* to SD100 Δ*sll0180* will not modify the intracellular or extracellular FFA concentrations.

5.1.2 Using complementation of *E. coli acrAB* mutants with *E. coil acrAB* to study the function of the AcrAB proteins

When *E. coli acrAB/emrAB* mutants were shown to lack the FFA secretion abilities of a wild-type *E. coli*, the researchers were able to add the wild-type *acrAB* genes back to the mutant *E. coli* and restore the FFA efflux function (21). In addition, when the *E. coli* AcrA protein was rendered nonfunctional using a deletion of the *E. coli acrA* gene, the ability of the AcrAB efflux pump to promote tolerance to Ery, Cm, tetracycline, crystal violet, ethidium bromide and other toxic chemicals in the *acrA* mutant *E. coli* strain was significantly decreased (140). The tolerance to the aforementioned chemicals was restored in the *acrA* deletion mutant *E. coli* cells was restored when the *E. coli* wild-type *acrA* gene was complemented into the *acrA* deficient strain using a plasmid vector containing the nucleotide sequence of *E. coli acrA* (152). The same restoration of tolerance to the numerous toxic chemicals mentioned previously was found in an *acrB* deficient *E. coli* strain when a plasmid containing *E. coli acrB* was conjugated into the *acrB* deficient strains (184).

I followed a similar complementation model using the following previously discussed mutant strains: SD100 Δ*slr2131*, SD277 Δ*slr2131*, SD100 Δ*sll0180*, and

SD277 Δ*sll0180*. However, instead of adding the cyanobacterial gene that was removed back to the mutant strain by way of a conjugative plasmid, I added the homologous *E. coli* gene, either *E. coli acrB* and *acrA*. *E. coli acrB* was added using a conjugative plasmid to transfer wild-type allele to the two strains containing *slr2131* mutations, SD100 Δ*slr2131* and SD277 Δ*slr2131*, while the wild-type *E. coli acrA* gene was added using a conjugative plasmid to transfer the wild-type gene to the two strains containing *sll0180* mutations, SD100 Δ*sll0180* and SD277 Δ*sll0180*.

5.2 Materials and Methods

5.2.1 Growth and culture conditions

The same culture and growth conditions were used for the research in this Chapter as described in Chapter 3.2.1 accompanied by the further detail of the sample preparation for mRNA analysis described in Chapter 4.2.1.

5.2.2 Strain Construction using Conjugation

Table 5.1 List of Plasmids Used for Complementation

Plasmid Name	Description	Parent	Curtiss Collec.	Reference
p*acrB*	Expression vector *E. coli acrB*	pΨ568	pΨ691	this research
pS*acrA*	Expression vector *E. coli acrA*	pΨ568	pΨ692	this research
pRL443	Helper plasmid with methylases	RP4	pRL443	(161)
pRL528	Conjugative plasmid	pRL542	pRL528	(161)

Table 5.2 List of Strains Used for Complementation

Strain Name	Description	Parent	Curtiss Collec.	Reference
SD100 △*slr2131* p*acrB*	SD100 with *slr2131* deleted with expression vector encoding *E. coli acrB*	SD100 Δ*slr2131*	SD665	this research
SD277 △*slr2131* p*acrB*	SD277 with *slr2131* deleted with expression vector encoding *E. coli acrB*	SD277 Δ*slr2131*	SD666	this research
SD100 △*sll0180* pS*acrA*	SD100 with *sll0180* deleted with expression vector encoding *E. coli acrA*	SD100 Δ*sll0180*	SD667	this research
SD277 △*sll0180* pS*acrA*	SD277 with *sll0180* deleted with expression vector encoding *E. coli acrA*	SD277 Δ*sll0180*	SD668	this research

I created a new plasmid for the research in this chapter, pS*acrA*. In Chapter 4, *E. coli acrA* was ligated in pKan, but I needed *acrA* on a vector with Spec and Str resistance because all four mutant strains are resistant to Kan. This new plasmid was created in the same protocol using the same primers from Table 4.1, including the combination of pS*acrA* and pRL528 into the same *E. coli* Top 10 cell as described in Chapter 4.2.2. The plasmids for this chapter are summarized in Table 5.1. Then pS*acrA* was conjugationally transferred into SD100 Δ*sll1080* and SD277 Δ*sll1080,* while *pacrB* was conjugationally transferred into both SD100 Δ*slr2131* and SD277 Δ*slr2131* using the conjugation procedure outline in Chapter 4.2.3. Briefly, 10 mL cultures of the mutant strain of cyanobacteria were grown to early log phase (OD_{730} of 0.4), while 15 mL cultures of *E. coli* Top 10 cells containing the expression vector with pRL528 were grown overnight in liquid LB containing appropriate antibiotics at 37ºC of the following strains alongside an *E. coli* culture containing pRL443. After washing each of the three cultures with BG-11, they were combined to allow the conjugation to proceed. After a 20-minute room-temperature incubation, 400 µL of the combination of the three cultures was placed on a nitrocellulose membrane that was resting on a BG-11 with 0.5% LB media added and incubated in the illuminated chamber (SANYO). A control combination of cultures was also used, in which no *E. coli* Top 10 cells with pRL443 were added to the other two cultures. Once a faint green color arose on the surface of the nitrocellulose membrane, the membrane was then transferred to a plate containing BG-11 with 40µg/mL of Kan and 30µg/mL each of Spec and Str to select for the presence p*acrB* or pS*acrA* and maintenance of the cassette conferring resistance for Kan in the cyanobacterial chromosomes that was exchanged for *slr2131* or *sll0180*. Individual colonies arose within

seven days and were picked off of the nitrocellulose membrane using a sterilized loop and patched onto a new BG-11 plate containing the aforementioned concentrations of Kan, Spec, and Str. The presence of the *E. coli* DNA and mRNA was tested using conventional PCR and RT-PCR as described in Chapters 4.2.3 and 4.2.4 using primers and plasmids highlighted in Table 4.1 and Table 4.2, respectively. The primers used for RT-PCR analysis are described in Table 4.4 for each of the two *E. coli* genes and the one SD100 gene used as a control in addition to *petB* from SD100 being used as an RNA control to ensure that RNA was being appropriately isolated. A conventional PCR was also performed using the DNase treated purified RNA samples from each of the strains described in this chapter. There was no DNA found in any RNA sample. The descriptions of resulting strains are summarized in Table 5.2.

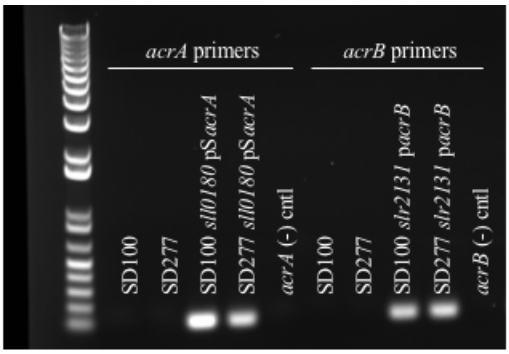


Figure 5.1 DNA Gel Electrophoresis displaying DNA Fragments from RT-PCR reactions of each the *E. coli* gene in the complementation strains

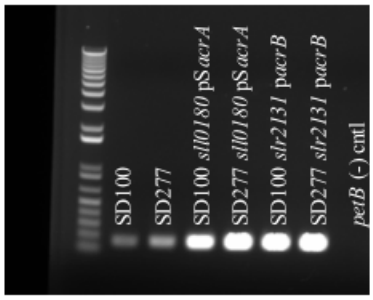


Figure 5.2 DNA Gel Electrophoresis displaying DNA Fragments from RT-PCR reactions of each the *petB* gene in the complementation strains as an RNA control

5.2.3 Tolerance Assays to determine half maximal inhibitory concentrations (IC_{50})

The same process was used in this chapter as was described in Chapter 3.2.4. But briefly, each of the strains was grown to a mid-log level growth phase. The cells were then diluted to an OD_{790} between 0.35 to 0.40 in 96-well plates (Corning) using various concentrations of Amp, R6G, Cm, and Ery, and grown for 3 days in the illuminated incubator (SANYO). The final OD_{790} was measured using a plate reader (Biotek). The measurements were analyzed using GraphPad Prism 6 software to determine the non-linear variable slope curve that best fits the regression of the concentration of cyanobacteria compared to the concentration of chemical, indicating their IC_{50} to the chemical.

5.2.4 FFA separation and gas chromatography measurements

The analysis of intracellular and extracellular FFA concentrations of each of the strains presented in this chapter follows the exact procedure from Chapter 3.2.5 by growing a 30 mL culture each strain to a final density of $8x10^8$ to $1.1x10^9$ cells/mL. Then the 20 mL of the culture of cyanobacteria was centrifuged, and the supernatant was removed from sample. The remaining pelleted cyanobacterial cells were resuspended in 3 mL of a 2:1 ratio of chloroform:methanol to follow the protocol of a modified Folch method for FFA extraction (117). Once the Folch method extracted sample was filtered and the resulting solvent was dried using a positive nitrogen evaporator, the sample was treated using the process of direct transesterification using the addition 10 mL hexane and 200 μL phosphoric acid. The addition 10 mL hexane and 200 μL phosphoric acid was also used to perform the process of transesterification in the supernatant collected from the initial centrifugation of the 20 mL culture. Each of the samples were processed using

a GC using nitrogen as the carrier gas. The peaks at specific time points are then analyzed for the area under each identified peak and comparing them to a series of standardized peaks.

5.3 Results and Discussion

5.3.1 Tolerance to Cm is restored in SD100 Δ*slr2131*, SD277 Δ*slr2131*, SD100 Δ*sll0180*, and SD277 Δ*sll0180* mutant strains when homologous *E. coli* gene is added relative to respective parent strain (SD100 or SD277).

Table 5.3 IC_{50} of Ampicillin, Chloramphenicol, Erythromycin, and Rhodamine 6G of SD277-Based Complementation Strains in which IC_{50} of derivative strain is significantly different than SD277 unless otherwise indicated with ns for not significant.

Treatment	SD277 µg/mL	SD277 Δ*slr2131* µg/mL	SD277 Δ*slr2131* p*acrB* µg/mL	SD277 Δ*sll0180* µg/mL	SD277 Δs*ll0180* pS*acrA* µg/mL
Amp	1570 ± 170	1001 ± 120	980.3 ± 37	450 ± 119	286.6 ± 113.8
R6G	1.41 ± 0.12	2.59 ± 0.12	1.48 ± 0.05 (ns)	0.94 ± 0.27	.18 ± .05
Cm	4.749 ± 1.043	1.873 ± 0.354	3.305 ± 1.100 (ns)	1.085 ± 0.589	3.486 ± 1.027 (ns)
Ery	0.427 ± 0.249	0.171 ± 0.044	0.095 ± 0.007	0.034 ± 0.007	0.075 ± 0.014

Restored tolerance is highlighted in green.

Table 5.4 IC_{50} of Ampicillin, Chloramphenicol, Erythromycin, and Rhodamine 6G of SD100-Based Complementation Strains in which IC_{50} of derivative strain is significantly different than SD100 unless otherwise indicated with ns for not significant.

Treatment	SD100 µg/mL	SD100 Δ*slr2131* µg/mL	SD100 Δ*slr2131* p*acrB* µg/mL	SD100 Δ*sll0180* µg/mL	SD100 Δs*ll0180* pS*acrA* µg/mL
Amp	955.2 ± 256	1280 ± 230 (ns)	891 ± 117 (ns)	598 ± 296	577.0 ± 23.2
R6G	6.08 ± .871	4.03 ± 0.43	3.94 ± 0.79	4.61 ± 0.51	1.80 ± .13
Cm	2.290 ± 0.401	0.586 ± 0.142	2.124 ± 1.004 (ns)	1.442 ± 0.206	4.73 ± 1.47
Ery	0.358 ± 0.081	0.054 ± 0.002	0.103 ± 0.019	0.172 ± 0.1	.144 ± .028

Restored tolerance is highlighted in green.

SD100 Δ*slr2131* and SD100 Δ*sll0180* possessed significantly lowered tolerances to Cm than did SD100 and similarly, and SD277 Δ*slr2131* and SD277 Δ*sll0180* possessed significantly lowered tolerances to Cm than did SDSD277 (Chapter 3). The tolerance of SD100 Δ*slr2131* p*acrB*, SD277 Δ*slr2131* p*acrB*, SD100 Δ*sll0180* pS*acrA*, and SD277 Δ*sll0180* pS*acrA* to Cm increased significantly compared to their parental (deletion mutant) strains to the point that each of the four complemented strain listed above are statistically the same as for SD100 and SD277 (Table 5.3 and Table 5.4). While, the IC_{50} of SD100 Δ*slr2131* p*acrB*, SD277 Δ*slr2131* p*acrB*, and SD277 Δ*sll0180* pS*acrA* did not surpass that of the respective control strains, SD100 and SD277, each had the equivalent tolerance to Cm as did SD100 and SD277 (Table 5.3 and Table 5.4). SD100 Δ*sll0180* pS*acrA*, on the other hand, did have a significantly greater tolerance to Cm when compared to SD100 (Table 5.4). SD277 Δ*slr2131* p*acrB* possessed a statistically unchanged IC_{50} to Amp and Ery when compared to SD277 Δ*slr2131* and both sets of strains had statistically lower tolerance to Amp and Ery when compared to SD277 (Table 5.3). When SD100 Δ*slr2131* p*acrB* was treated with Ery, it possessed a significantly greater IC_{50} when compared to SD100 Δ*slr2131*, but still statistically less than SD100 (Table 5.4). SD277 Δ*sll0180* pS*acrA* possessed significantly decreased tolerances to Amp and R6G but an increased tolerance to Ery relative to SD277 Δ*sll0180* (Table 5.3). SD100 Δ*sll0180* pS*acrA* also had a decreased tolerance to R6G but the same tolerance to Amp and Ery relative to SD100 Δ*sll1080* (Table 5.4). SD100 Δ*slr2131* p*acrB* had statistically the same IC_{50} when treated with increasing concentrations of R6G as did SD100 Δ*slr2131*, both of which are significantly less than SD100 (Table 5.4). p*acrB* did

not restore R6G tolerance in the SD100 Δ*slr2131* mutant strain. SD277 Δ*slr2131* had an increased tolerance to R6G; when SD277 Δ*slr2131* was complemented with the wild-type *E. coli acrB* allele, the tolerance of the resulting strain fell to statistically identical levels as observed for SD277 (Table 5.3). All of the expected *E. coli* genes were expressed in the appropriate strains (Figure 5.1), while SD100/SD277 *petB* mRNA was also observed using RT-PCR analysis for all strains (Figure 5.2).

Neither the lack of expression of the *E. coli* gene in the cyanobacterial strain nor the growth rate of the strain provides any insight into why the tolerance to Ery, Amp, and R6G is not significantly improved when a wild-type *E. coli* gene are added to the four mutant strains of SD100 and SD277. The one consistent piece of information drawn for the IC_{50} assays in this chapter is that the tolerance to Cm can be restored by the addition of homologous *E. coli* genes to SD100 and SD277 strains lacking native *sll0180* and *slr2131* genes. The remaining data does not indicate that the proteins encoded by *acrA* and *acrB* have a substantial role in conferring tolerance to toxic chemicals in mutant strains of SD100 and SD277 when *sll0180* or *slr2131* are removed from the cyanobacteria. This could be a result of the inability of AcrA or AcrB to interact with native proteins involved in transport within *Synechocystis* sp. PCC 6803. For example, AcrA may not function with Slr1270 (TolC homolog) and Slr2131 (AcrB homolog) to facilitate the movement of the antibiotics or R6G from Slr2131 to the Slr1270 outer membrane duct as seen in *E. coli* where the structure and interactions of proteins is critical to the observed phenotype of an operational AcrAB-TolC efflux pump (141, 148, 185, 186). This potential lack of an interaction of AcrA with native Slr2131 and Slr1270 is investigated further in the following section.

5.3.2 The addition of *acrB* increases FFA secretion

Figure 5.3 Extracellular Free Fatty Acid Concentrations of the SD277-Based Deletion and Complementation Strains where ** is p < 0.005, **** p < 0.0001, and ns is not significant

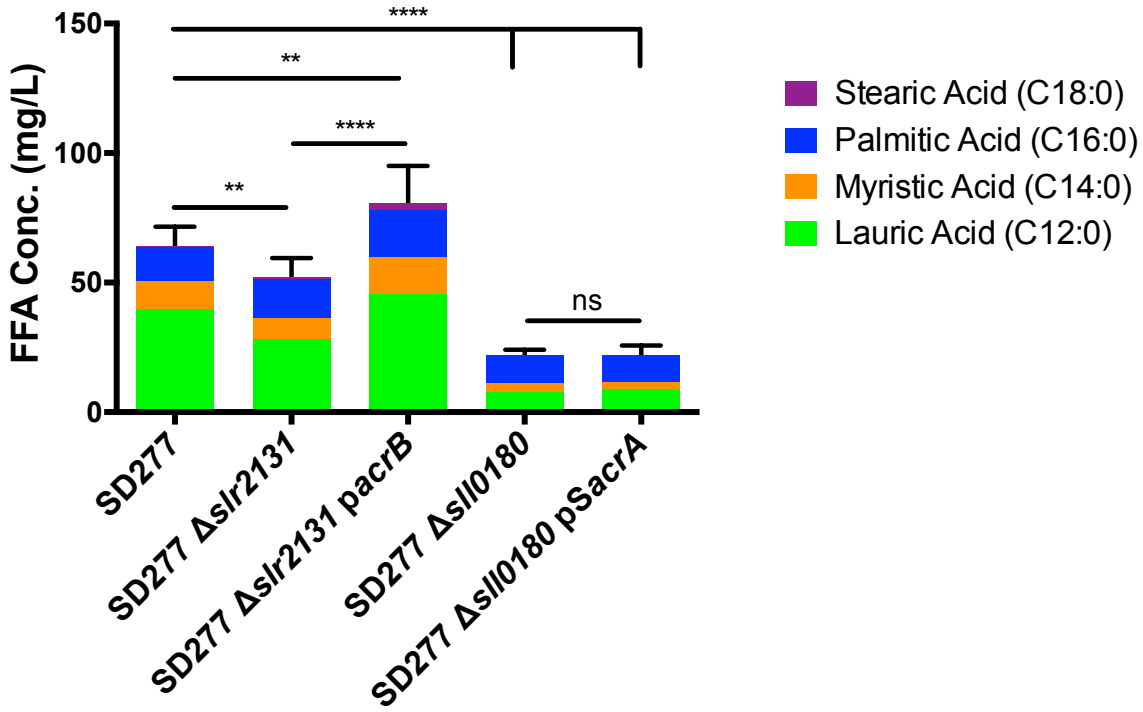


Figure 5.4 Intracellular Free Fatty Acid Concentrations of the SD277-Based Deletion and Complementation Strains where * is p < 0.05, *** is p < 0.0005, **** p < 0.0001, and ns is not significant

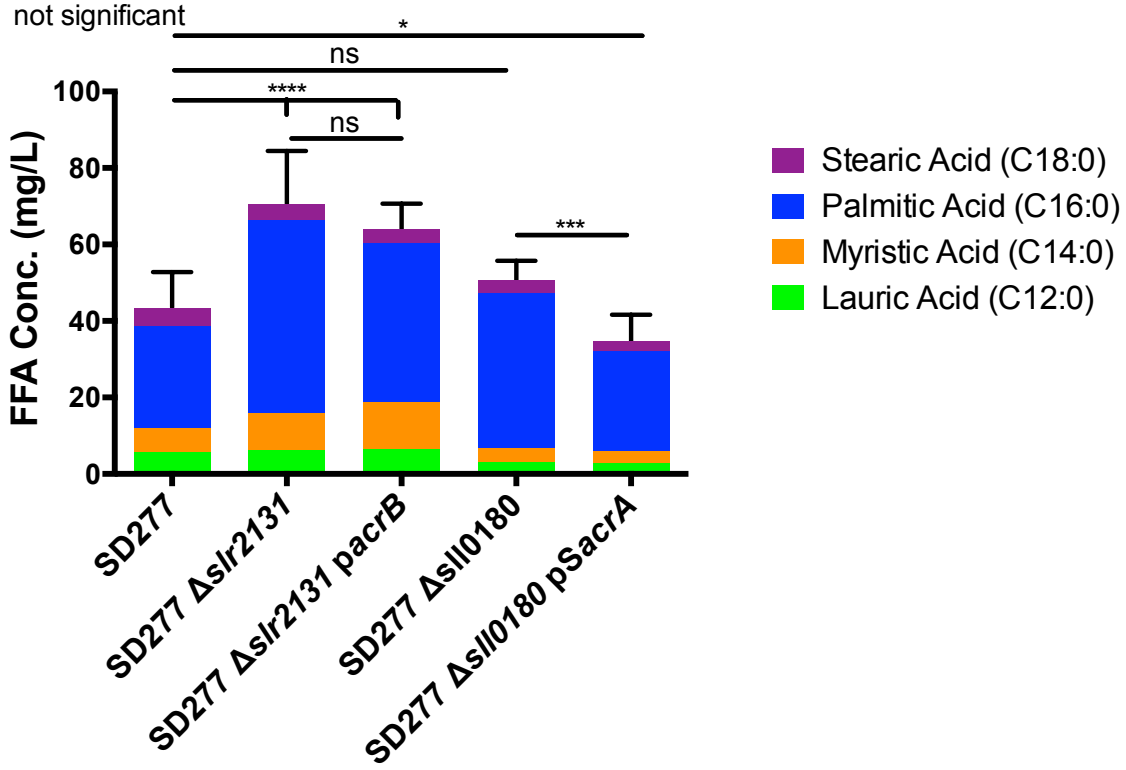


Figure 5.5 Extracellular Free Fatty Acid Concentrations of the SD100-Based Deletion and Complementation Strains where * is $p < 0.05$ ** is $p < 0.005$, and ns is not significant

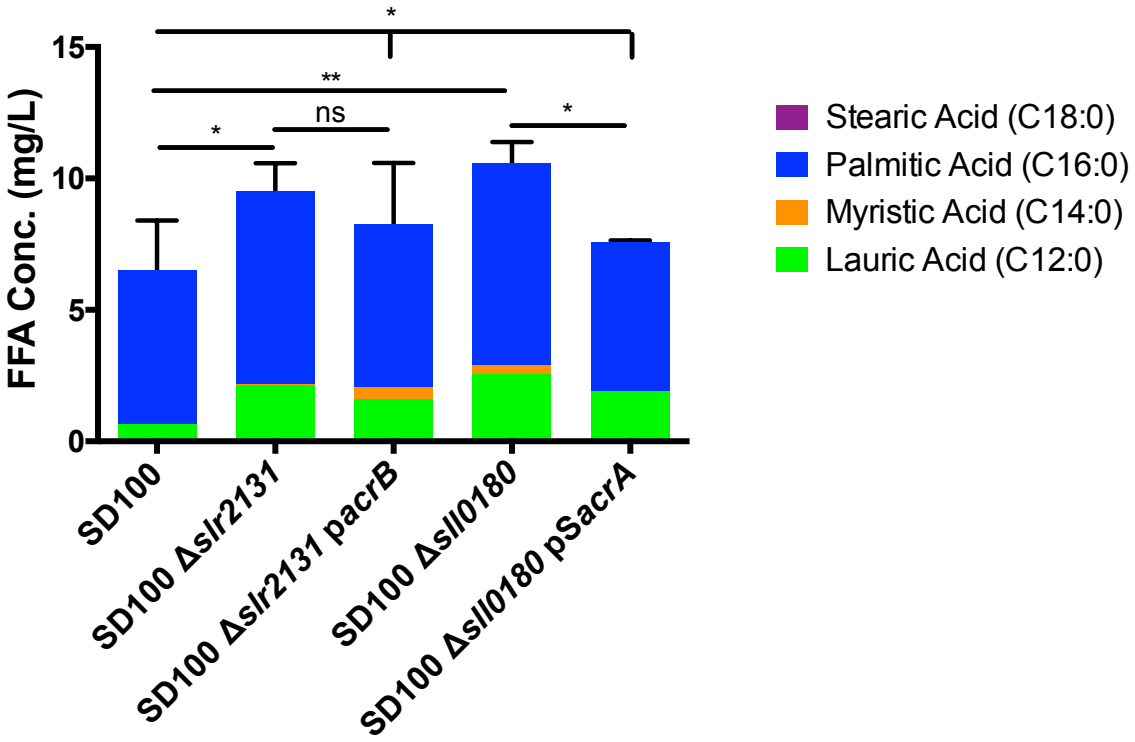
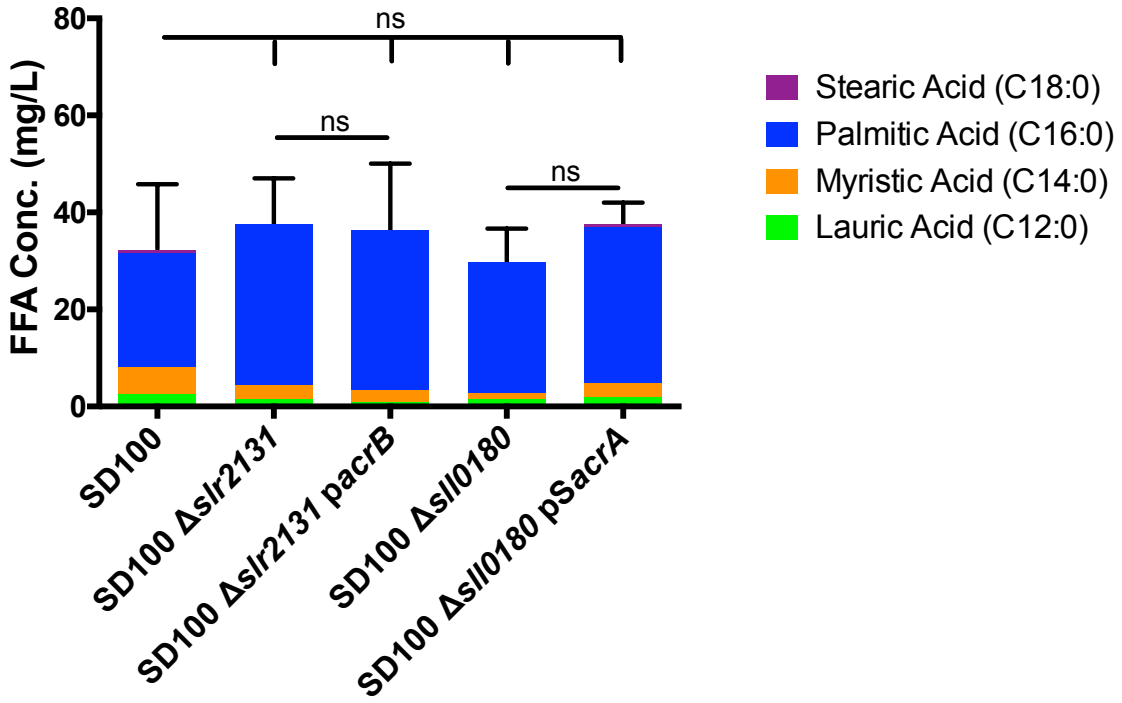


Figure 5.6 Intracellular Free Fatty Acid Concentrations of the SD100-Based Deletion and Complementation Strains where ns is not significant



SD277 Δ*slr2131* p*acrB* possessed a significantly greater extracellular and intracellular FFA concentration compared to SD277 (Figure 5.3). SD277 Δ*slr2131* p*acrB* also possessed a greater concentration of extracellular FFAs compared SD277 Δ*slr2131*, but the same concentration of intracellular FFAs. SD277 Δ*sll0180* p*acrA* possessed the same concentration of extracellular FFA compared to SD277 Δ*sll0180*, so both possessed significantly less extracellular FFA concentration compared to SD277. SD277 Δ*sll0180* p*acrA* had a decreased intracellular FFA concentration relative to SD277 Δ*sll0180* while there was no difference between the intracellular FFA concentrations of SD277 Δ*slr2131* p*acrB* and SD277 Δ*slr2131* (Figure 5.4). The addition of p*acrA* to SD277 Δ*sll0180* did assist in FFA efflux, but the opposite was true when p*acrB* was added to SD277

Δ*slr2131*. The addition of wild-type *E. coli* gene *acrA* and *acrB* to SD100 Δ*sll0180* and SD100 Δ*slr2131*, respectively, either decreased the extracellular FFA concentration relative to the mutant strain or the concentration remained the same (Figure 5.5). None of the intracellular FFA concentrations of SD100 and its derivatives varied (Figure 5.6). None of the mutant or complemented strains have any significant variation in growth patterns relative to their respective SD100 or SD277 parent strains (Figure 5.7 and Figure 5.8). All of the expected *E. coli* genes were expressed in the appropriate strains (Figure 5.1), while SD100/SD277 *petB* mRNA was also observed using RT-PCR analysis for all strains (Figure 5.2).

SD277 Δ*slr2131* p*acrB* possessed the highest concentration of extracellular FFAs of any strain in this research. The addition of the wild-type *E. coli acrB* to SD277 Δ*slr2131* counteracted the detrimental effects observed in the extracellular FFA concentration of SD277 Δ*slr2131*. The extracellular FFA of SD277 Δ*slr2131* p*acrB* was so high that it possessed a significant increase of extracellular FFA concentration compared to SD277. If Slr2131 is a multidrug efflux pump, responsible for FFA efflux, a deletion of the gene encoding that pump (*slr2131*) would almost certainly cause a decrease in extracellular FFA concentrations (Figure 5.3). Furthermore, the wild-type *E. coli acrB* allele was present in the cell, as revealed by synthesis of mRNA (Figure 5.1). Data from Chapter 4 shows that the addition of p*acrB* to SD277 does not increase the extracellular FFA concentration, but the increase observed here must be due to the presence of the *E. coli* AcrB protein in the membrane of SD277, exchanging protons for FFA substrates. As was seen in Chapter 4, the addition of the parent plasmid of p*acrB*, pSpec, did not increase the FFA concentration of SD277 therefore, the increase seen here

can only be attributed to the encoded AcrB protein in SD277 Δ*slr2131* p*acrB*. Interestingly when only p*acrB* was added to SD277 there was not an increase in extracellular FFA but there was when p*acrB* was added to SD277 Δ*slr2131* relative to SD277, indicating a number of conclusions. First, the native Slr2131 efflux pump must not be in place for the optimal operation of *E. coli* AcrB, likely because AcrB uses the same Slr1270 outer membrane duct as Slr2131 would use in its native configuration. There have been previous experiments indicating the role of Slr1270 and the likelihood that it functions in a similar manner to TolC (60, 61). This is all important because the results indicate that *E. coli* AcrB can work with Slr1270 to increase extracellular FFA concentrations through FFA export. This opens up an interesting opportunity for future research to look into how to further promote FFA efflux and production by modifying native transport systems or introducing exogenous ones now that it's clear that a native active efflux system is used to move FFA out of *Synechocystis* sp. PCC 6803 derived strains' cells and exogenous genes can be used to compensate for the loss-of-function created by removing the native active efflux system.

Conversely, the addition *E. coli acrA* was not able to compensate for the loss-of-function with respect to FFA export of SD277 Δ*sll0180*. *E. coli* AcrA may just not effectively recognize binding sites on the Slr1270 outer membrane duct or there may exist differences in the membrane structure of these cyanobacteria that may not be ideal or even necessitate AcrA. AcrA is created from 397 amino acids while Sll0180 is composed of 501 amino acids. When comparing the homology of the two proteins, the first 131 amino acids of Sll0180 share an 18% identity to AcrA and, similarly, nearly the last 262 amino acids of Sll0180 share a 19% identity to AcrA (Sll0180 amino acids 500

and 501 were not included in this identity analysis) (110). But this leaves the gap from amino acids 132 to 237 in Sll0180, bearing virtually no homology to AcrA. When identifying possible protein binding regions in these 105 nonhomologous amino acids, two are predicted at sites 132 and 183. AcrA does not possess this central region that is present in Sll0180 and the region may affect the ability of AcrA to bind to a protein within the membranes of *Synechocystis* sp. PCC 6803. In addition, previous research has supported the hypothesis that the periplasmic end of the Slr1270 outer membrane duct in *Synechocystis* sp. PCC 6803 remains open without the MFP, which is not the case for *E. coli* TolC lacking an MFP. Without *E. coli* AcrA or a different MFP, the periplasmic end of *E. coli* TolC collapses, while Slr1270 maintains its open at both ends of the duct without an MFP (60). Based on the results of the *sll0180* mutant strains, the Sll0180 MFP is necessary for a native efflux mechanism, but the resulting loss of function cannot be compensated through the addition of *E.* coli AcrA in SD100 or SD277 *sll0180* mutant strains. The compounding differences between Sll0180 and AcrA including the amino acid sequence lengths (501 compared to 397), nonhomologous protein binding sites, and the different roles each may have in relation to the outer membrane duct may all lead to the inability for *E. coli* AcrA to restore the loss of function demonstrated in *sll0180* mutant SD100 and SD277 strains with respect to FFA secretion and tolerance to Amp, Cm, and Ery.

Figure 5.7 Growth Curve of SD277-Based Deletion and Complementation Strains

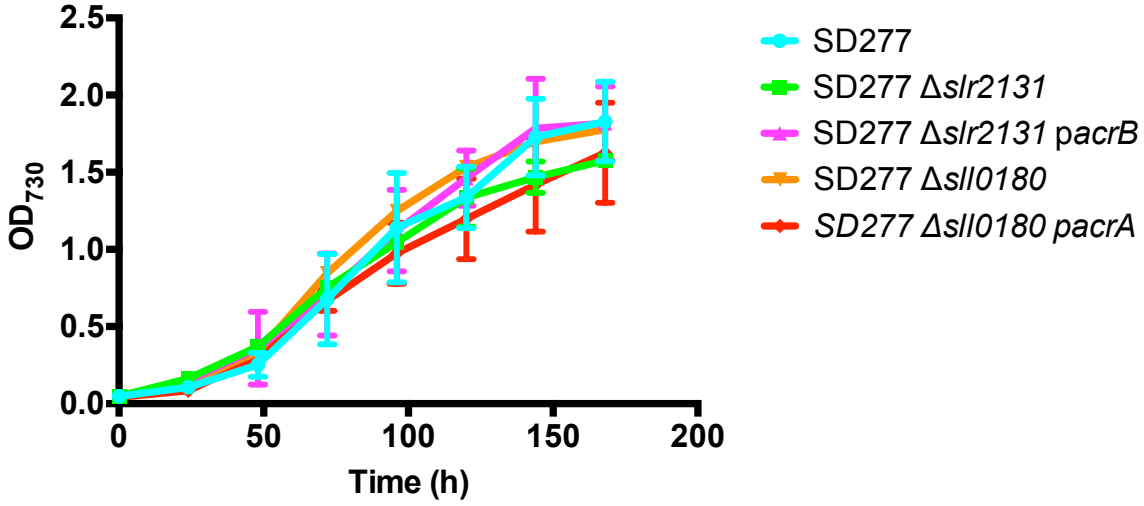
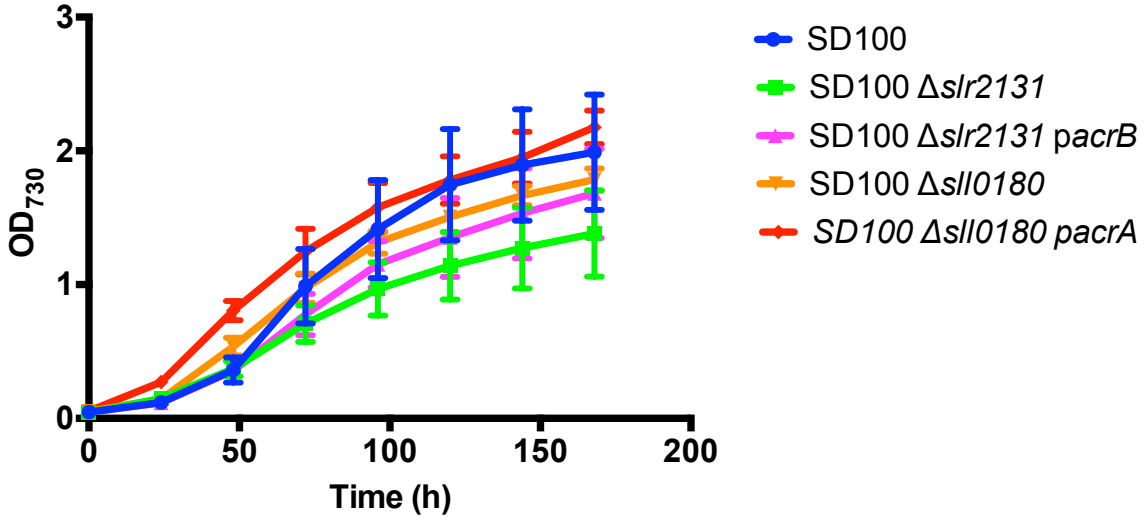


Figure 5.8 Growth Curve of SD100-Based Deletion and Complementation Strains



5.4 Conclusion

The tolerances of SD100 Δ*slr2131* p*acrB*, SD277 Δ*slr2131* p*acrB*, SD100 Δ*sll0180* pS*acrA* and SD277 Δ*sll0180* pS*acrA* to Cm were equivalent to the tolerance of each strain's respective parent strain, either SD100 or SD277, to Cm. The tolerances of SD100

Δ*slr2131* p*acrB*, SD277 Δ*slr2131* p*acrB*, SD100 Δ*sll0180* pS*acrA* and SD277 Δ*sll0180* pS*acrA* to R6G, Ery, and Amp were not restored to equivalent levels as observed in SD100 or SD277. SD277 Δ*slr2131* p*acrB* had a significantly greater extracellular FFA concentration when compared to SD277 and SD277 Δ*slr2131*. The intracellular FFA concentrations of SD277 Δ*slr2131* p*acrB* and SD277 Δ*slr2131* remain higher than SD277 but statistically equivalent to each other. This piece of data leads me to the conclusion that Slr2131 is a multidrug efflux pump that can be complemented with *E. coli acrB*. AcrB can function in *Synechocystis* sp. PCC 6803 to move FFA out of the cell to increase the extracellular FFA concentrations of SD277 Δ*slr2131* p*acrB* relative to SD277. This finding should lead to future studies regarding the transport of industrially relevant chemicals in cyanobacterial strains genetically modified to produce such chemicals at greater rates than the wild-type strain.

With the use of SD277, genetically modified to make a high concentration of FFA, and SD100 I was able to demonstrate some of the roles of Sll0180 and Slr2131. These roles included the ability to secrete Amp, Cm, Ery, and FFAs. In addition, wild-type *E. coli acrB* allele can be added to SD277 Δ*slr2131* to restore the extracellular FFA concentration that was observed in SD277.

CHAPTER 6

EVALUATION AND FURTHERANCE OF RESEARCH PROGRESS

6.1 Research progress and future goals

I spent a great deal of time piecing together this research project, identifying the *E. coli* AcrAB-TolC system for its abilities to secrete FFA and other related hydrocarbons including hexane. I then combed the SD100 genome for homologous sequences and was pleased to find *slr1270* and *slr2131* relatively quickly that are homologous to *E. coli tolC* and *E. coli acrB*, respectively. The homologous element to *E. coli acrA* took far more time to piece together. There was (and still is) limited research identifying MFPs in SD100, but there are a few proteomic studies and nucleotide sequence comparisons investigating two genes conferring putative MFPs SD100 *sll1053* and *sll0180*. I choose to investigate the role of SD100 *sll0180* after comparing the nucleotide sequences of *sll1053* and *sll0180* to *E. coli acrA*. In addition, numerous researchers were able to show that the protein encoded by *sll0180* existed the membrane of SD100 (20, 59, 99, 100). The data from Chapter 3 helps elucidate that Sll0180 and Slr2131 are both required for FFA secretion in SD277, a high FFA producing strain created by genetically modifying SD100. The four mutant strains nearly universally had a decrease of tolerance to four chemicals used as treatments. Sll0180 is likely an MFP associated with a multidrug efflux pump, and Slr2131 is likely a multidrug efflux pump.

In Chapter 4, I added each of the three *E. coli* genes that encoded the AcrAB-TolC efflux to expression vectors, pKan and pSpec. Both the DNA and mRNA of each of the *E. coli* genes was identified in both SD100 and SD277. The research in this section did not provide data illustrating the increase in extracellular FFA concentrations when any of

the *E. coli* genes were added to SD277. Unfortunately, the addition of the plasmids to SD277 decreased extracellular FFA concentrations in some cases, but the addition of a combination of wild-type *E. coli acrA* and wild-type *E. coli tolC* or wild-type *E. coli acrA* and wild-type *E. coli acrB* to SD277 created strains with higher extracellular FFA concentrations relative to SD277 pSpec pKan. So, while I still do not understand why the addition of plasmids would decrease extracellular FFA concentrations, I am interested in the facts that intracellular FFA concentrations do not decrease and growth rates are not changed by the addition of the plasmids. As discussed, the LexA homolog in SD100 (and SD277) may have a role in modulating FFA synthesis with respect to the addition of exogenous plasmids.

My final task was to complement the *sll0180* and *slr2131* mutations made in Chapter 3. The four complemented strains had their tolerance to Cm restored to concentrations equivalent to their respective parent strains. The SD277 Δ*slr2131* p*acrB* strain achieved the greatest total extracellular FFA concentration, significantly greater than the extracellular FFA concentration observed in SD277.

To continue this research, I would immediately begin placing each of these wild-type *E. coli* genes at neutral sites in the *Synechocystis* sp. PCC 6803 chromosome, which have been described previously (187). One example of a neutral site in an intergenic region identified between *slr2060* and *sll1956*, in which an exogenous gene could replace this site with likely no phenotypic differences observed. I would also remove the *lexA* homolog, *sll1626*, from SD277 and SD100 to investigate the role of the protein encoded by this gene on both FFA transport across the membrane and tolerance to toxic

chemicals. In particular, I would want to see if SD277 Δ*sll1626* decreased extracellular FFA concentrations when exogenous plasmids were added to the cells.

Additionally, both *slr2131* and *sll0180* should be ligated into pSpec. Then these two newly created plasmids should be transformed into their respective SD100 and SD277 deletion strains to see if the loss of function observed in Chapter 3, with respect to the tolerance to antibiotics and FFA efflux, can be reversed. This would provide additional insight into how Slr2131 and Sll0180 function with respect to efflux. A reversal of the loss of function would provide more evidence for the function of Slr2131 and Sll0180 established by my research. Furthermore, I could also use an inhibitor to hydrogen/proton antiporter efflux, such as MBX2319, which has been shown to inhibit that activity of the AcrAB efflux system in *E. coli* (188). If the extracellular concentrations of FFA in SD277 is decreased significantly, this would further indicate that Slr2131 and Sll0180 are responsible for a significant portion of FFA efflux, as opposed to passive transport. Passive transport would be demonstrated if the addition of MBX2319 did not significantly decrease the SD277 extracellular concentration of FFA.

6.2 Applications to future cyanobacterial chemical production

I wanted to increase the secretion of FFAs from continually growing cyanobacteria to foster an efficient FFA production process, in which the FFA extraction step is removed from the conventional processing of bacteria for FFAs. After reading a paper published by two of my committee members, Dr. Nielsen and Dr. Rittmann, the idea flourished. They, along with numerous co-authors, compare the effectiveness of various ways to get laurate out of the culture media without destroying the cells or requiring the

use of an energy intensive centrifugation to pellet the cells to allow removal of the supernatant (189). My initial idea was to filter or centrifuge for separation of the cells from the growth media, but this group of engineers presented a much more practical idea. This system of using FFA absorbents has the ability to gradually accumulate secreted FFA that my strains are able to produce.

slr2131 possesses similar genetic characteristics to that of *E. coli acrB*. The encoded protein of *slr2131*, Slr2131, share many functions to *E. coli* AcrB as well. Slr2131 extrudes antibiotics and FFA, both consistent with the function of AcrB. The same comparisons can be said of *sll0180* and its encoded Sll0180 protein relative to *E. coli acrA* and its encoded AcrA protein. This research also provides evidence that both Sll0180 and Slr2131 bear some responsibility in tolerance to antibiotics and secretion of FFA. The removal of these proteins may have other effects in other genetically modified strains of SD100 including those that are engineered to produce significantly greater concentration of ethanol; since ethanol must be secreted to prevent cell death (43).

I was not anticipating the deleterious phenotypes observed in strains with the addition of pKan and pSpec. SD100 carries 7 plasmids itself of varying sizes, from 2.3kb to 120kb (190–194). Previous research uses of expression vectors to encode exogenous genes to increase biofuel production. Researchers encoded genes to promote the production of isoprene in *Synechocystis* sp. PCC 6803 (35). Isoprene production increased as a result of the genetic modifications, but the researchers did not note an altered isoprene production due to the addition of control plasmids lacking exogenous genes.

But many of the problems observed in this research can be readily addressed in

future research on biofuel production, but we have to have a consistent need for these alternative fuels. Without the need, there will not be the funding to support the research.

6.3 Biofuels in a broader context

Initially, research investigating cyanobacteria to produce biofuels seems counterintuitive. Using *E. coli*, one can produce far greater concentrations of FFAs than any photosynthetic bacteria can produce. However, *E. coli* while requiring a feedstock of sugars, *E. coli* grow fast, can synthesize nearly every exogenous gene using an expression vector or engineered into its chromosome, and the ability to produce, but not secrete FFAs in a continuously growing culture. *E. coli* can produce 400-times the concentration of FFAs compared to SD277 or the newly created SD277 Δ*slr2131* p*acrB* strains, but the *Enterobacteriaceae* will never be able to solve the far greater problem of getting CO_2 out of the atmosphere. While using cyanobacterial based biofuels may just get the carbon sequestered temporarily, more research into producing fuels from renewable sources that include bacteria may lead to further discoveries that prove to be invaluable to prevent the Atlantic Ocean from creeping the 8 feet up to the door of Tampa's newest condominium complex (195). Much of the biofuel research now focuses on identifying these optimal feedstocks like sawgrass or corn (196), but I argue that some of this time and energy be spent optimizing biofuels in photosynthetic organisms. I know that the green bacteria grow more slowly, are more difficult to genetically manipulate, and require more love and attention to grow at peak rates, but they have to possibility of helping to counteract the effects of the burning of fossil fuels.

I want to create biofuels from photosynthetic organisms using atmospheric carbon

dioxide as the source of the backbone of these hydrocarbons, sequestering it away from the accumulation of greenhouse gases even if it is only temporary. The most substantial problem is that gas prices have remained low for a decade. In 2006, gas prices were above $4 a gallon around the country for months and people did not ever see that price decreasing. People dumped their pickups and SUVs in favor of 4-cylinder family sedans, taking advantage of the Cash for Clunkers program designed to boost the American economy; the economic boost never came to fruition (197). As gas prices dropped, people hopped right back to the trucks they love. Hydraulic fracturing of oil shales has contributed to decreased gas prices as the United States is now the largest producer of natural gas (https://www.eia.gov/dnav/ng/hist/n9070us2M.htm). With mounting concerns over the resultant wastewater pollution and earthquakes that the pressurized liquids cause, hydraulic fracturing is filled with unsustainable protocols (198). Cyanobacterial fuels including ethanol and diesel may be a future part of the solution to present environmental issues with fracturing and rising greenhouse gas emissions, but there exists a current market for various photosynthetic organisms as dietary supplements, food additives, salads, and animal feeds.

The cyanobacterium, *Arthrospira platensis*, known for its vitamin B_{12} content and high protein percentage relative to total cell dry weight (199), is added under the name Spirulina to many green smoothies. The cyanobacteria are always the last ingredient, which usually means that it is present in the smallest quantity. As any person that has been around cyanobacteria long enough has noticed the potent odor (200) from dense, established cultures often described as “fishy,” which may be one of the reasons for the small quantity used in each smoothie. The popularity of protein powders, shakes,

smoothies, and other supplements is a multi-billion-dollar market, presenting large market for cyanobacterial protein but they do come with consumption risks (201). Other companies have begun marketing lipids produced in these photosynthetic organisms as dietary supplements including omega-3 fatty acids because of their established health benefits (202).

I think the world is heading towards electrified, self-driving cars that will initially be powered by batteries but will eventually be replaced by a wireless charging system on the road. The electricity will be derived predominantly from solar and wind energy and stored in massive batteries on-site or within the grid. The future of biofuels may be limited to commercial airline travel, which, for the time being, has no feasible plans to electrify. Maybe one day we will drive around our classic cars from today on biofuels, but until then the most responsible venture is to facilitate any reduction of greenhouse gases out of the atmosphere and into a sink. I hope it includes photosynthetic organisms and that this research provides some new insight into the efflux mechanisms that may help make this possible.

REFERENCES

1. Brocks JJ. 1999. Archean Molecular Fossils and the Early Rise of Eukaryotes. Science 285:1033–1036.

2. Campbell IH, Allen CM. 2008. Formation of supercontinents linked to increases in atmospheric oxygen. Nat Geosci 1:554–558.

3. Kopp RE, Kirschvink JL, Hilburn IA, Nash CZ. 2005. The Paleoproterozoic snowball Earth: A climate disaster triggered by the evolution of oxygenic photosynthesis. Proc Natl Acad Sci 102:11131–11136.

4. Dismukes GC, Klimov VV, Baranov SV, Kozlov YN, DasGupta J, Tyryshkin A. 2001. The origin of atmospheric oxygen on Earth: The innovation of oxygenic photosynthesis. Proc Natl Acad Sci 98:2170–2175.

5. White D, Thiessen R. 1913. The Origin of Coal. US Government Printing Office.

6. Berl E. 1937. Origin of Petroleum, p. 1–18. *In* . American Institute of Mining and Metallurgical Engineers, Oklahoma City.

7. World Meteorological Organisation. 2017. WMO statement on the state of the global climate in 2016.

8. Searchinger Ti, Heimlich R, Houghton RA, Dong F, Elobeid A, Fabiso J, Tokgoz S, Hayes D, Yu T-H. 2008. Use of U.S. Croplands for Biofuels Increases Greenhouse Gases through Emissions from Land-Use Change. Am Assoc Adv Sci 319:1238–1240.

9. Post WM, Kwon KC. 2008. Soil carbon sequestration and land-use change: processes and potential. Glob Change Biol 6:317–327.

10. Teixeira ACR, Sodré JR, Guarieiro LLN, Vieira ED, de Medeiros FF, Alves CT. 2016. A Review on Second and Third Generation Bioethanol Production.

11. Hill J, Nelson E, Tilman D, Polasky S, Tiffany D. 2006. Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. Proc Natl Acad Sci 103:11206–11210.

12. Pimentel D. 2003. Ethanol Fuels: Energy Balance, Economics, and Environmental Impacts Are Negative. Nat Resour Res 8.

13. Grima EM, Belarbi E-H, Fernández FA, Medina AR, Chisti Y. 2003. Recovery of microalgal biomass and metabolites: process options and economics. Biotechnol Adv 20:491–515.

14. Liu X, Sheng J, Curtiss III R. 2011. Fatty acid production in genetically modified cyanobacteria. Proc Natl Acad Sci 108:6899–6904.

15. Yu Y, You L, Liu D, Hollinshead W, Tang Y, Zhang F. 2013. Development of Synechocystis sp. PCC 6803 as a Phototrophic Cell Factory. Mar Drugs 11:2894–2916.

16. Wang B, Pugh S, Nielsen DR, Zhang W, Meldrum DR. 2013. Engineering cyanobacteria for photosynthetic production of 3-hydroxybutyrate directly from CO_2. Metab Eng 16:68–77.

17. Baebprasert W, Jantaro S, Khetkorn W, Lindblad P, Incharoensakdi A. 2011. Increased H2 production in the cyanobacterium Synechocystis sp. strain PCC 6803 by redirecting the electron supply via genetic engineering of the nitrate assimilation pathway. Metab Eng 13:610–616.

18. Wang W, Liu X, Lu X. 2013. Engineering cyanobacteria to improve photosynthetic production of alka(e)nes. Biotechnol Biofuels 6:69.

19. Lee D-G, Kwon J, Eom C-Y, Kang Y-M, Roh SW, Lee K-B, Choi J-S. 2015. Directed analysis of cyanobacterial membrane phosphoproteome using stained phosphoproteins and titanium-enriched phosphopeptides. J Microbiol 53:279–287.

20. Pisareva T, Shumskaya M, Maddalo G, Ilag L, Norling B. 2007. Proteomics of *Synechocystis* sp PCC 6803: identification of novel integral plasma membrane proteins. FEBS J 274:791–804.

21. Lennen RM, Politz MG, Kruziki MA, Pfleger BF. 2013. Identification of transport proteins involved in free fatty acid efflux in *Escherichia coli*. J Bacteriol 195:135–144.

22. Nakamura H. 1965. Gene-controlled resistance to acriflavine and other basic dyes in *Escherichia coli*. J Bacteriol 90:8–14.

23. Gonçalves CF, Pacheco CC, Tamagnini P, Oliveira P. 2018. Identification of inner membrane translocase components of TolC-mediated secretion in the cyanobacterium *Synechocystis* sp PCC 6803. Environ Microbiol 20:2354–2369.

24. Kaneko T, Tabata S. 1997. Complete genome structure of the unicellular cyanobacterium Synechocystis sp. PCC6803. Plant Cell Physiol 38:1171–1176.

25. Kaneko T, Sato S, Kotani H, Tanaka A, Asamizu E, Nakamura Y, Miyajima N, Hirosawa M, Sugiura M, Sasamoto S, others. 1996. Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp strain PCC 6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Res 3:109–136.

26. Nozzi NE, Oliver JWK, Atsumi S. 2013. Cyanobacteria as a Platform for Biofuel Production. Front Bioeng Biotechnol 1.

27. Ikeuchi M, Tabata S. 2001. Synechocystis sp. PCC 6803—a useful tool in the study of the genetics of cyanobacteria. Photosynth Res 70:73–83.

28. Clark DJ, Maaløe O. 1967. DNA replication and the division cycle in Escherichia coli. J Mol Biol 23:99–112.

29. Labarre J, Chauvat F, Thuriaux P. 1989. Insertional mutagenesis by random cloning of antibiotic resistance genes into the genome of the cyanobacterium Synechocystis strain PCC 6803. J Bacteriol 171:3449–3457.

30. Griese M, Lange C, Soppa J. 2011. Ploidy in cyanobacteria. FEMS Microbiol Lett 323:124–131.

31. Lu X, Vora H, Khosla C. 2008. Overproduction of free fatty acids in E. coli: Implications for biodiesel production. Metab Eng 10:333–339.

32. Kothari A, Parameswaran P, Garcia-Pichel F. 2014. Powerful fermentative hydrogen evolution of photosynthate in the cyanobacterium Lyngbya aestuarii BL J mediated by a bidirectional hydrogenase. Front Microbiol 5.

33. Cournac L, Guedeney G, Peltier G, Vignais PM. 2004. Sustained Photoevolution of Molecular Hydrogen in a Mutant of Synechocystis sp. Strain PCC 6803 Deficient in the Type I NADPH-Dehydrogenase Complex. J Bacteriol 186:1737–1746.

34. Gao Z, Zhao H, Li Z, Tan X, Lu X. 2012. Photosynthetic production of ethanol from carbon dioxide in genetically engineered cyanobacteria. Energy Environ Sci 5:9857–9865.

35. Lindberg P, Park S, Melis A. 2010. Engineering a platform for photosynthetic isoprene production in cyanobacteria, using Synechocystis as the model organism. Metab Eng 12:70–79.

36. Atsumi S, Higashide W, Liao JC. 2009. Direct photosynthetic recycling of carbon dioxide to isobutyraldehyde. Nat Biotechnol 27:1177–1180.

37. Varman AM, Xiao Y, Pakrasi HB, Tang YJ. 2013. Metabolic Engineering of Synechocystis sp. Strain PCC 6803 for Isobutanol Production. Appl Environ Microbiol 79:908–914.

38. Qi F, Yao L, Tan X, Lu X. 2013. Construction, characterization and application of molecular tools for metabolic engineering of Synechocystis sp. Biotechnol Lett 35:1655–1661.

39. Ungerer J, Tao L, Davis M, Ghirardi M, Maness P-C, Yu J. 2012. Sustained Photosynthetic Conversion of Atmospheric CO2 to Ethylene in Recombinant Cyanobacterium Synechocystis 6803.

40. Angermayr SA, Paszota M, Hellingwerf KJ. 2012. Engineering a Cyanobacterial Cell Factory for Production of Lactic Acid. Appl Environ Microbiol 78:7098–7106.

41. Dunlop MJ. 2011. Engineering microbes for tolerance to next-generation biofuels. Biotechnol Biofuels 4:32.

42. Fu P. 2009. Genome-scale modeling of Synechocystis sp. PCC 6803 and prediction of pathway insertion. J Chem Technol Biotechnol 84:473–483.

43. Zhang Y, Niu X, Shi M, Pei G, Zhang X, Chen L, Zhang W. 2015. Identification of a transporter Slr0982 involved in ethanol tolerance in cyanobacterium Synechocystis sp. PCC 6803. Front Microbiol 6.

44. Teixeira MC, Raposo LR, Mira NP, Lourenco AB, Sa-Correia I. 2009. Genome-Wide Identification of Saccharomyces cerevisiae Genes Required for Maximal Tolerance to Ethanol. Appl Environ Microbiol 75:5761–5772.

45. Linton KJ, Higgins CF. 1998. The *Escherichia coli* ATP-binding cassette (ABC) proteins. Mol Microbiol 28:5–13.

46. Prosecka J, Orlov AV, Fantin YS, Zinchenko VV, Babykin MM, Tichy M. 2009. A novel ATP-binding cassette transporter is responsible for resistance to viologen herbicides in the cyanobacterium Synechocystis sp. PCC 6803. FEBS J 276:4001–4011.

47. Giner-Lamia J, Robles-Rengel R, Hernández-Prieto MA, Muro-Pastor MI, Florencio FJ, Futschik ME. 2017. Identification of the direct regulon of NtcA during early acclimation to nitrogen starvation in the cyanobacterium Synechocystis sp. PCC 6803. Nucleic Acids Res 45:11800–11820.

48. Tahara H, Uchiyama J, Yoshihara T, Matsumoto K, Ohta H. 2012. Role of Slr1045 in environmental stress tolerance and lipid transport in the cyanobacterium Synechocystis sp. PCC6803. Biochim Biophys Acta BBA - Bioenerg 1817:1360–1366.

49. Tahara H, Matsuhashi A, Uchiyama J, Ogawa S, Ohta H. 2015. Sll0751 and Sll1041 are involved in acid stress tolerance in Synechocystis sp. PCC 6803. Photosynth Res 125:233–242.

50. Fisher ML, Allen R, Luo Y, Curtiss R. 2013. Export of Extracellular Polysaccharides Modulates Adherence of the Cyanobacterium Synechocystis. PLoS ONE 8:e74514.

51. Mikkat S, Hagemann M. 2000. Molecular analysis of the ggtBCD gene cluster of Synechocystis sp. strain PCC6803 encoding subunits of an ABC transporter for osmoprotective compounds. Arch Microbiol 174:273–282.

52. Eisenhut M, Bauwe H, Hagemann M. 2007. Glycine accumulation is toxic for the cyanobacterium Synechocystis sp. strain PCC 6803, but can be compensated by supplementation with magnesium ions. FEMS Microbiol Lett 277:232–237.

53. Ongley SE, Pengelly JJL, Neilan BA. 2016. A multidrug efflux response to methyl viologen and acriflavine toxicity in the cyanobacterium Synechocystis sp. PCC6803. J Appl Phycol 28:2793–2803.

54. Paulsen IT, Chen J, Nelson KE, Saier, Jr. MH. 2001. Comparative genomics of microbial drug efflux systems. J Mol Microbiol Biotechnol 3:145–150.

55. Zhu H, Ren X, Wang J, Song Z, Shi M, Qiao J, Tian X, Liu J, Chen L, Zhang W. 2013. Integrated OMICS guided engineering of biofuel butanol-tolerance in photosynthetic Synechocystis sp. PCC 6803. Biotechnol Biofuels 6:106.

56. Ruffing AM, Trahan CA. 2014. Biofuel toxicity and mechanisms of biofuel tolerance in three model cyanobacteria. Algal Res 5:121–132.

57. Sinetova MA, Los DA. 2016. New insights in cyanobacterial cold stress responses: Genes, sensors, and molecular triggers. Biochim Biophys Acta BBA - Gen Subj 1860:2391–2403.

58. Morona R, Manning PA, Reeves P. 1983. Identification and characterization of the TolC protein, an outer membrane protein from Escherichia coli. J Bacteriol 153:693–699.

59. Huang F, Hedman E, Funk C, Kieselbach T, Schröder WP, Norling B. 2004. Isolation of outer membrane of *Synechocystis* sp. PCC 6803 and its proteomic characterization. Mol Cell Proteomics 3:586–595.

60. Agarwal R, Zakharov S, Hasan SS, Ryan CM, Whitelegge JP, Cramer WA. 2014. Structure–function of cyanobacterial outer-membrane protein, Slr1270: homolog of *Escherichia coli* drug export/colicin import protein, TolC. FEBS Lett 588:3793–3801.

61. Oliveira P, Martins NM, Santos M, Pinto F, Büttel Z, Couto NAS, Wright PC, Tamagnini P. 2016. The versatile TolC-like Slr1270 in the cyanobacterium *Synechocystis* sp PCC 6803. Environ Microbiol 18:486–502.

62. Escherich T. 1885. Sitzungsberichte. Gesellschaft für Morphologie und Physiologie.

63. Nakamura H. 1968. Genetic determination of resistance to acriflavine, phenethyl alcohol, and sodium dodecyl sulfate in Escherichia coli. J Bacteriol 96:987–996.

64. Swick MC, Morgan-Linnell SK, Carlson KM, Zechiedrich L. 2011. Expression of multidrug efflux pump genes *acrAB-tolC*, *mdfA*, and *norE* in *Escherichia coli*

clinical isolates as a function of fluoroquinolone and multidrug resistance. Antimicrob Agents Chemother 55:921–924.

65. Kim HB, Wang M, Park CH, Kim E-C, Jacoby GA, Hooper DC. 2009. oqxAB Encoding a Multidrug Efflux Pump in Human Clinical Isolates of Enterobacteriaceae. Antimicrob Agents Chemother 53:3582–3584.

66. Okusu H, Ma D, Nikaido H. 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. J Bacteriol 178:306–308.

67. Murakami S, Nakashima R, Yamashita E, Yamaguchi A. 2003. Crystal structure of bacterial multi-drug efflux transporter AcrB. Nihon Kessho Gakkaishi 45:256–261.

68. Chang G, Roth CB. 2001. Structure of MsbA from E. coli: A homolog of the multidrug resistance ATP binding cassette (ABC) transporters. Science 293:1793–1800.

69. Li X-Z, Nikaido H. 2009. Efflux-Mediated Drug Resistance in Bacteria: An Update. Drugs 69:1555–1623.

70. Qosa H, Miller DS, Pasinelli P, Trotti D. 2015. Regulation of ABC efflux transporters at blood-brain barrier in health and neurological disorders. Neuroprotection Basic Mech Transl Potential 1628:298–316.

71. Borths EL, Poolman B, Hvorup RN, Locher KP, Rees DC. 2005. In Vitro Functional Characterization of BtuCD-F, the Escherichia coli ABC Transporter for Vitamin B12 Uptake. Biochemistry 44:16301–16309.

72. Zhou Z, White KA, Polissi A, Georgopoulos C, Raetz CRH. 1998. Function of Escherichia coli MsbA, an Essential ABC Family Transporter, in Lipid A and Phospholipid Biosynthesis. J Biol Chem 273:12466–12475.

73. Naroditskaya V, Schlosser MJ, Fang NY, Lewis K. 1993. An E. coli Gene emrD Is Involved in Adaptation to Low Energy Shock. Biochem Biophys Res Commun 196:803–809.

74. Krulwich TA, Quirk PG, Guffanti AA. 1990. Uncoupler-resistant mutants of bacteria. Microbiol Rev 54:52–65.

75. Nishino K, Yamaguchi A. 2001. Analysis of a complete library of putative drug transporter genes in *Escherichia coli*. J Bacteriol 183:5803–5812.

76. Ninio S, Rotem D, Schuldiner S. 2001. Functional Analysis of Novel Multidrug Transporters from Human Pathogens. J Biol Chem 276:48250–48256.

77. Kaatz GW, McAleese F, Seo SM. 2005. Multidrug Resistance in Staphylococcus aureus Due to Overexpression of a Novel Multidrug and Toxin Extrusion (MATE) Transport Protein. Antimicrob Agents Chemother 49:1857–1864.

78. Tikhonova EB, Zgurskaya HI. 2004. AcrA, AcrB, and TolC of Escherichia coli Form a Stable Intermembrane Multidrug Efflux Complex. J Biol Chem 279:32116–32124.

79. Kobayashi K, Tsukagoshi N, Aono R. 2001. Suppression of Hypersensitivity of Escherichia coli acrB Mutant to Organic Solvents by Integrational Activation of the acrEF Operon with the IS1 or IS2 Element. J Bacteriol 183:2646–2653.

80. Sulavik MC, Houseweart C, Cramer C, Jiwani N, Murgolo N, Greene J, DiDomenico B, Shaw KJ, Miller GH, Hare R, Shimer G. 2001. Antibiotic Susceptibility Profiles of Escherichia coli Strains Lacking Multidrug Efflux Pump Genes. Antimicrob Agents Chemother 45:1126–1136.

81. Schielke S, Schmitt C, Spatz C, Frosch M, Schubert-Unkmeir A, Kurzai O. 2010. The Transcriptional Repressor FarR Is Not Involved in Meningococcal Fatty Acid Resistance Mediated by the FarAB Efflux Pump and Dependent on Lipopolysaccharide Structure. Appl Environ Microbiol 76:3160–3169.

82. Hocquet D, Nordmann P, El Garch F, Cabanne L, Plesiat P. 2006. Involvement of the MexXY-OprM Efflux System in Emergence of Cefepime Resistance in Clinical Strains of Pseudomonas aeruginosa. Antimicrob Agents Chemother 50:1347–1351.

83. Gutierrez O, Juan C, Cercenado E, Navarro F, Bouza E, Coll P, Perez JL, Oliver A. 2007. Molecular Epidemiology and Mechanisms of Carbapenem Resistance in Pseudomonas aeruginosa Isolates from Spanish Hospitals. Antimicrob Agents Chemother 51:4329–4335.

84. Hoiczyk E, Hansel A. 2000. Cyanobacterial cell walls: news from an unusual prokaryotic envelope. J Bacteriol 182:1191–1199.

85. Niederholtmeyer H, Wolfstadter BT, Savage DF, Silver PA, Way JC. 2010. Engineering Cyanobacteria To Synthesize and Export Hydrophilic Products. Appl Environ Microbiol 76:3462–3466.

86. Núñez MF, Kwon O, Wilson TH, Aguilar J, Baldoma L, Lin ECC. 2002. Transport of l-Lactate, d-Lactate, and Glycolate by the LldP and GlcA Membrane Carriers of Escherichia coli. Biochem Biophys Res Commun 290:824–829.

87. Núñez MF, Teresa Pellicer M, Badía J, Aguilar J, Baldomà L. 2001. The gene yghK linked to the glc operon of Escherichia coli encodes a permease for glycolate that is structurally and functionally similar to L-lactate permease. Microbiology 147:1069–1077.

88. Zaslavskaia LA, Lippmeier JC, Shih C, Ehrhardt D, Grossman AR, Apt KE. 2001. Trophic Conversion of an Obligate Photoautotrophic Organism Through Metabolic Engineering. Science 292:2073.

89. McEwen JT, Machado IMP, Connor MR, Atsumi S. 2013. Engineering Synechococcus elongatus PCC 7942 for Continuous Growth under Diurnal Conditions. Appl Environ Microbiol 79:1668–1675.

90. Kucho K, Okamoto K, Tsuchiya Y, Nomura S, Nango M, Kanehisa M, Ishiura M. 2005. Global Analysis of Circadian Expression in the Cyanobacterium Synechocystis sp. Strain PCC 6803. J Bacteriol 187:2190–2199.

91. Contreras F-X, Sánchez-Magraner L, Alonso A, Goñi FM. 2010. Transbilayer (flip-flop) lipid motion and lipid scrambling in membranes. Front Membr Biochem 584:1779–1786.

92. Nakano M, Fukuda M, Kudo T, Matsuzaki N, Azuma T, Sekine K, Endo H, Handa T. 2009. Flip-Flop of Phospholipids in Vesicles: Kinetic Analysis with Time-Resolved Small-Angle Neutron Scattering. J Phys Chem B 113:6745–6748.

93. Paulsen IT, Brown MH, Skurray RA. 1996. Proton-Dependent Multidrug Efflux Systems. MICROBIOL REV 60:575–608.

94. Giner-Lamia J, Lopez-Maury L, Reyes JC, Florencio FJ. 2012. The CopRS Two-Component System Is Responsible for Resistance to Copper in the Cyanobacterium Synechocystis sp. PCC 6803. PLANT Physiol 159:1806–1818.

95. Giner-Lamia J, López-Maury L, Florencio FJ. 2014. Global Transcriptional Profiles of the Copper Responses in the Cyanobacterium Synechocystis sp. PCC 6803. PLoS ONE 9:e108912.

96. Gittins JR. 2015. Cloning of a copper resistance gene cluster from the cyanobacterium Synechocystis sp. PCC 6803 by recombineering recovery. FEBS Lett 589:1872–1878.

97. Zhao S, Wang X, Niu G, Dong W, Wang J, Fang Y, Lin Y, Liu L. 2016. Structural basis for copper/silver binding by the Synechocystis metallochaperone CopM. Acta Crystallogr Sect Struct Biol 72:997–1005.

98. López-Maury L, Giner-Lamia J, Florencio FJ. 2012. Redox control of copper homeostasis in cyanobacteria. Plant Signal Behav 7:1712–1714.

99. Huang F, Parmryd I, Nilsson F, Persson AL, Pakrasi HB, Andersson B, Norling B. 2002. Proteomics of *Synechocystis* sp strain PCC 6803: identification of plasma membrane proteins. Mol Cell Proteomics 1:956–966.

100. Pisareva T, Kwon J, Oh J, Kim S, Ge C, Wieslander Å, Choi J-S, Norling B. 2011. Model for membrane organization and protein sorting in the cyanobacterium *Synechocystis* sp PCC 6803 inferred from proteomics and multivariate sequence analyses. J Proteome Res 10:3617–3631.

101. Liu J, Chen L, Wang J, Qiao J, Zhang W. 2012. Proteomic analysis reveals resistance mechanism against biofuel hexane in Synechocystis sp. PCC 6803. Biotechnol Biofuels 5:68.

102. White DG, Goldman JD, Demple B, Levy SB. 1997. Role of the acrAB locus in organic solvent tolerance mediated by expression of marA, soxS, or robA in Escherichia coli. J Bacteriol 179:6122–6126.

103. Needleman SB, Wunsch CD. 1970. A general method applicable to the search for similarities in the amino acid sequence of two proteins. J Mol Biol 48:443–453.

104. Battistuzzi FU, Feijao A, Hedges SB. 2004. A genomic timescale of prokaryote evolution: insights into the origin of methanogenesis, phototrophy, and the colonization of land. BMC Evol Biol 4:44–44.

105. Battistuzzi FU, Hedges SB. 2009. A Major Clade of Prokaryotes with Ancient Adaptations to Life on Land. Mol Biol Evol 26:335–343.

106. Sheridan PP, Freeman KH, Brenchley JE. 2003. Estimated Minimal Divergence Times of the Major Bacterial and Archaeal Phyla. Geomicrobiol J 20:1–14.

107. Kumar S, Stecher G, Suleski M, Hedges SB. 2017. TimeTree: A Resource for Timelines, Timetrees, and Divergence Times. Mol Biol Evol 34:1812–1819.

108. Ochman H, Wilson AC. 1987. Evolution in bacteria: Evidence for a universal substitution rate in cellular genomes. J Mol Evol 26:74–86.

109. Šorfová P, Škeříková A, Hypša V. 2008. An effect of 16S rRNA intercistronic variability on coevolutionary analysis in symbiotic bacteria: Molecular phylogeny of Arsenophonus triatominarum. Syst Appl Microbiol 31:88–100.

110. Rost B, Yachdav G, Liu J. 2004. The PredictProtein server. Nucleic Acids Res 32:W321–W326.

111. Cho H, Cronan Jr. JE. 1995. Defective Export of a Periplasmic Enzyme Disrupts Regulation of Fatty Acid Synthesis. J Biol Chem 270:4216–4219.

112. Allen MM, Stanier RY. 1968. Selective isolation of blue-green algae from water and soil. Microbiology 51:203–209.

113. Miller J. 1972. Experiments in molecular genetics / Jeffrey H. Miller, 3rd, illustrated ed. Cold Spring Harbor Laboratory.

114. Lagarde D, Beuf L, Vermaas W. 2000. Increased Production of Zeaxanthin and Other Pigments by Application of Genetic Engineering Techniques toSynechocystis sp. Strain PCC 6803. Appl Environ Microbiol 66:64–72.

115. Liu X, Curtiss R. 2009. Nickel-inducible lysis system in *Synechocystis* sp PCC 6803. Proc Natl Acad Sci 106:21550–21554.

116. Grigorieva G, Shestakov S. 1982. Transformation in the cyanobacterium Synechocystis sp 6803. FEMS Microbiol Lett 13:367–370.

117. Sheng J, Vannela R, Rittmann BE. 2011. Evaluation of methods to extract and quantify lipids from *Synechocystis* PCC 6803. Bioresour Technol 102:1697–1703.

118. Folch J, Lees M, Sloane-Stanley GH, others. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497–509.

119. Lalman JA, Bagley DM. 2004. Extracting long-chain fatty acids from a fermentation medium. J Am Oil Chem Soc 81:105–110.

120. Ge Q, Yamada Y, Zgurskaya H. 2009. The C-Terminal Domain of AcrA Is Essential for the Assembly and Function of the Multidrug Efflux Pump AcrAB-TolC. J Bacteriol 191:4365–4371.

121. Pradel E, Pages J-M. 2002. The AcrAB-TolC Efflux Pump Contributes to Multidrug Resistance in the Nosocomial Pathogen Enterobacter aerogenes. Antimicrob Agents Chemother 46:2640–2643.

122. Łęski TA, Tomasz A. 2005. Role of Penicillin-Binding Protein 2 (PBP2) in the Antibiotic Susceptibility and Cell Wall Cross-Linking of Staphylococcus aureus: Evidence for the Cooperative Functioning of PBP2, PBP4, and PBP2A. J Bacteriol 187:1815–1824.

123. Yarborough JM. 1974. cw dye laser emission spanning the visible spectrum. Appl Phys Lett 24:629–630.

124. Modica-Napolitano JS, Aprille JR. 1987. Basis for the Selective Cytotoxicity of Rhodamine 123. Cancer Res 47:4361.

125. Bartsevich VV, Pakrasi HB. 1999. Membrane Topology of MntB, the Transmembrane Protein Component of an ABC Transporter System for Manganese in the CyanobacteriumSynechocystis sp. Strain PCC 6803. J Bacteriol 181:3591–3593.

126. Badarau A, Firbank SJ, Waldron KJ, Yanagisawa S, Robinson NJ, Banfield MJ, Dennison C. 2008. FutA2 Is a ferric binding protein from *Synechocystis* PCC 6803. J Biol Chem 283:12520–12527.

127. Katoh H, Hagino N, Ogawa T. 2001. Iron-binding activity of FutA1 aubunit of an ABC-type iron transporter in the cyanobacterium *Synechocystis* sp Strain PCC 6803. Plant Cell Physiol 42:823–827.

128. Koropatkin N, Randich AM, Bhattacharyya-Pakrasi M, Pakrasi HB, Smith TJ. 2007. The structure of the iron-binding protein, FutA1, from *Synechocystis* 6803. J Biol Chem 282:27468–27477.

129. Tahara H, Fukai S, Sambe M, Kobayashi M, Uchiyama J, Ohta H. 2013. Characterization of the ABC Transporter Gene slr1045 Involved in Acid-stress Tolerance of Synechocystis sp. PCC 6803, p. 596–598. *In* Kuang, T, Lu, C, Zhang, L (eds.), Photosynthesis Research for Food, Fuel and the Future. Springer Berlin Heidelberg.

130. Sanglard D, Ischer F, Monod M, Bille J. 1997. Cloning of Candida albicans genes conferring resistance to azole antifungal agents: characterization of CDR2, a new multidrug ABC transporter gene. Microbiology 143:405–416.

131. Kowata H, Tochigi S, Takahashi H, Kojima S. 2017. Outer Membrane Permeability of Cyanobacterium Synechocystis sp. Strain PCC 6803: Studies of Passive Diffusion of Small Organic Nutrients Reveal the Absence of Classical Porins and Intrinsically Low Permeability. J Bacteriol 199:e00371-17.

132. Hansel A, Pattus F, Jürgens UJ, Tadros MH. 1998. Cloning and characterization of the genes coding for two porins in the unicellular cyanobacterium Synechococcus PCC 6301. Biochim Biophys Acta BBA - Gene Struct Expr 1399:31–39.

133. Terry N. 1980. Limiting Factors in Photosynthesis. Plant Physiol 65:114.

134. Nestorovich EM, Danelon C, Winterhalter M, Bezrukov SM. 2002. Designed to penetrate: Time-resolved interaction of single antibiotic molecules with bacterial pores. Proc Natl Acad Sci U S A 99:9789–9794.

135. Yoshimura F, Nikaido H. 1985. Diffusion of beta-lactam antibiotics through the porin channels of Escherichia coli K-12. Antimicrob Agents Chemother 27:84–92.

136. Waxman DJ, Strominger JL. 1983. Penicillin-Binding Proteins and the Mechanism of Action of Beta-Lactam Antibiotics1. Annu Rev Biochem 52:825–869.

137. Grinter NJ, Barth PT. 1976. Characterization of SmSu plasmids by restriction endonuclease cleavage and compatibility testing. J Bacteriol 128:394–400.

138. Scherzinger E, Haring V, Lurz R, Otto S. 1991. Plasmid RSF1010 DNA replication in vitro promoted by purified RSF1010 RepA, RepB and RepC proteins. Nucleic Acids Res 19:1203–1211.

139. de Zwaig RN, Luria SE. 1967. Genetics and physiology of colicin-tolerant mutants of *Escherichia coli*. J Bacteriol 94:1112–1123.

140. Ma D, Cook DN, Alberti M, Pon NG, Nikaido H, Hearst JE. 1993. Molecular cloning and characterization of *acrA* and *acrE* genes of *Escherichia coli*. J Bacteriol 175:6299–6313.

141. Touzé T, Eswaran J, Bokma E, Koronakis E, Hughes C, Koronakis V. 2004. Interactions underlying assembly of the Escherichia coli AcrAB-TolC multidrug efflux system: Multidrug efflux system assembly. Mol Microbiol 53:697–706.

142. Pos KM. 2009. Drug transport mechanism of the AcrB efflux pump. Biochim Biophys Acta BBA - Proteins Proteomics 1794:782–793.

143. Seeger MA, Schiefner A, Eicher T, Verrey F, Diederichs K, Pos KM. 2006. Structural Asymmetry of AcrB Trimer Suggests a Peristaltic Pump Mechanism. Science 313:1295–1298.

144. Gu R, Li M, Su C-C, Long F, Routh MD, Yang F, McDermott G, Yu EW. 2008. Conformational change of the AcrR regulator reveals a possible mechanism of induction. Acta Crystallograph Sect F Struct Biol Cryst Commun 64:584–588.

145. Murakami S, Nakashima R, Yamashita E, Matsumoto T, Yamaguchi A. 2006. Crystal structures of a multidrug transporter reveal a functionally rotating mechanism. Nature 443:173–179.

146. Seeger MA, von Ballmoos C, Eicher T, Brandstätter L, Verrey F, Diederichs K, Pos KM. 2008. Engineered disulfide bonds support the functional rotation mechanism of multidrug efflux pump AcrB. Nat Struct 38 Mol Biol 15:199–205.

147. Schulz R, Vargiu AV, Collu F, Kleinekathöfer U, Ruggerone P. 2010. Functional Rotation of the Transporter AcrB: Insights into Drug Extrusion from Simulations. PLoS Comput Biol 6.

148. Koronakis V, Sharff A, Koronakis E, Luisi B, Hughes C. 2000. Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. Nature 405:914–919.

149. Wandersman C, Delepelaire P. 1990. TolC, an Escherichia coli outer membrane protein required for hemolysin secretion. Proc Natl Acad Sci 87:4776–4780.

150. Symmons MF, Bokma E, Koronakis E, Hughes C, Koronakis V. 2009. The assembled structure of a complete tripartite bacterial multidrug efflux pump. Proc Natl Acad Sci 106:7173–7178.

151. Mikolosko J, Bobyk K, Zgurskaya HI, Ghosh P. 2006. Conformational Flexibility in the Multidrug Efflux System Protein AcrA. Structure 14:577–587.

152. Elkins CA, Nikaido H. 2003. Chimeric Analysis of AcrA Function Reveals the Importance of Its C-Terminal Domain in Its Interaction with the AcrB Multidrug Efflux Pump. J Bacteriol 185:5349–5356.

153. Stegmeier JF, Polleichtner G, Brandes N, Hotz C, Andersen C. 2006. Importance of the Adaptor (Membrane Fusion) Protein Hairpin Domain for the Functionality of Multidrug Efflux Pumps †. Biochemistry 45:10303–10312.

154. Davis MS, Solbiati J, Cronan JE. 2000. Overproduction of Acetyl-CoA Carboxylase Activity Increases the Rate of Fatty Acid Biosynthesis in Escherichia coli. J Biol Chem 275:28593–28598.

155. Heath RJ, Rock CO. 1996. Regulation of Fatty Acid Elongation and Initiation by Acyl-Acyl Carrier Protein in Escherichia coli. J Biol Chem 271:1833–1836.

156. Lennen RM, Braden DJ, West RM, Dumesic JA, Pfleger BF. 2010. A Process for Microbial Hydrocarbon Synthesis: Overproduction of Fatty Acids in Escherichia coli and Catalytic Conversion to Alkanes. Biotechnol Bioeng 106:10.1002/bit.22660.

157. Lennen RM, Kruziki MA, Kumar K, Zinkel RA, Burnum KE, Lipton MS, Hoover SW, Ranatunga DR, Wittkopp TM, Marner WD, Pfleger BF. 2011. Membrane Stresses Induced by Overproduction of Free Fatty Acids in Escherichia coli. Appl Environ Microbiol 77:8114–8128.

158. Ruffing AM, Jones HDT. 2012. Physiological effects of free fatty acid production in genetically engineered Synechococcus elongatus PCC 7942. Biotechnol Bioeng 109:2190–2199.

159. Sarcina M, Murata N, Tobin MJ, Mullineaux CW. 2003. Lipid diffusion in the thylakoid membranes of the cyanobacterium Synechococcus sp.: effect of fatty acid desaturation. FEBS Lett 553:295–298.

160. Ruffing AM. 2014. Improved Free Fatty Acid Production in Cyanobacteria with Synechococcus sp. PCC 7002 as Host. Front Bioeng Biotechnol 2:17.

161. Elhai J, Vepritskiy A, Muro-Pastor AM, Flores E, Wolk CP. 1997. Reduction of conjugal transfer efficiency by three restriction activities of *Anabaena* sp strain PCC 7120. J Bacteriol 179:1998–2005.

162. Camsund D, Heidorn T, Lindblad P. 2014. Design and analysis of LacI-repressed promoters and DNA-looping in a cyanobacterium. J Biol Eng 8:4.

163. Garvin RT, Biswas DK, Gorini L. 1974. The Effects of Streptomycin or Dihydrostreptomycin Binding to 16S RNA or to 30S Ribosomal Subunits. Proc Natl Acad Sci U S A 71:3814–3818.

164. Burns DJW, Cundliffe E. 1973. Bacterial-Protein Synthesis. Eur J Biochem 37:570–574.

165. Misumi M, Tanaka N. 1980. Mechanism of inhibition of translocation by kanamycin and viomycin: A comparative study with fusidic acid. Biochem Biophys Res Commun 92:647–654.

166. Moazed D, Noller HF. 1987. Interaction of antibiotics with functional sites in 16S ribosomal RNA. Nature 327:389.

167. Elhai J, Wolk CP. 1988. Conjugal transfer of DNA to cyanobacteria, p. 747–754. *In* Methods in Enzymology. Academic Press.

168. Haase J, Lurz R, Grahn AM, Bamford DH, Lanka E. 1995. Bacterial conjugation mediated by plasmid RP4: RSF1010 mobilization, donor-specific phage propagation, and pilus production require the same Tra2 core components of a proposed DNA transport complex. J Bacteriol 177:4779–4791.

169. Pansegrau W, Lanka E, Barth PT, Figurski DH, Guiney DG, Haas D, Helinski DR, Schwab H, Stanisich VA, Thomas CM. 1994. Complete Nucleotide Sequence of Birmingham IncPα Plasmids: Compilation and Comparative Analysis. J Mol Biol 239:623–663.

170. Pinto F, Pacheco CC, Ferreira D, Moradas-Ferreira P, Tamagnini P. 2012. Selection of suitable reference genes for RT-qPCR analyses in cyanobacteria. PLoS ONE 7:e34983.

171. Cheah UE, Weigand WA, Stark BC. 1987. Effects of recombinant plasmid size on cellular processes in Escherichia coli. Plasmid 18:127–134.

172. Honda Y, Sakai H, Hiasa H, Tanaka K, Komano T, Bagdasarian M. 1991. Functional division and reconstruction of a plasmid replication origin: molecular dissection of the oriV of the broad-host-range plasmid RSF1010. Proc Natl Acad Sci 88:179–183.

173. Seo Jin-Ho, Bailey James E. 1985. Effects of recombinant plasmid content on growth properties and cloned gene product formation in Escherichia coli. Biotechnol Bioeng 27:1668–1674.

174. Summers DK, Sherratt DJ. 1984. Multimerization of high copy number plasmids causes instability: Cole 1 encodes a determinant essential for plasmid monomerization and stability. Cell 36:1097–1103.

175. Lenhart J, Schroeder J, Walsh B, Simmons L. 2012. DNA repair and genome maintenance in *Bacillus subtilis* 76:530–564.

176. Ayumi K, Akihito K, Kosuke T, Yasushi T, Yoshitaka N, Yukako H. 2017. The LexA transcription factor regulates fatty acid biosynthetic genes in the cyanobacterium *Synechocystis* sp PCC 6803. Plant J 92:189–198.

177. Francis D, Laetitia H, Franck C, Corinne C-C. 2004. Function and regulation of the cyanobacterial genes *lexA*, *recA,* and *ruvB*: LexA is critical to the survival of cells facing inorganic carbon starvation. Mol Microbiol 53:65–80.

178. Patterson-Fortin LM, Colvin KR, Owttrim GW. 2006. A LexA-related protein regulates redox-sensitive expression of the cyanobacterial RNA helicase, crhR. Nucleic Acids Res 34:3446–3454.

179. Oliveira P, Lindblad P. 2011. Novel insights into the regulation of LexA in the cyanobacterium *Synechocystis* sp strain PCC 6803. J Bacteriol 193:3804–3814.

180. Raczkowska A, Trzos J, Lewandowska O, Nieckarz M, Brzostek K. 2015. Expression of the AcrAB Components of the AcrAB-TolC Multidrug Efflux Pump of Yersinia enterocolitica Is Subject to Dual Regulation by OmpR. PLoS ONE 10:1–22.

181. Meng X, Shang H, Zheng Y, Zhang Z. 2013. Free fatty acid secretion by an engineered strain of Escherichia coli. Biotechnol Lett 35:2099–2103.

182. Xu P, Gu Q, Wang W, Wong L, Bower AGW, Collins CH, Koffas MAG. 2013. Modular optimization of multi-gene pathways for fatty acids production in E. coli. Nat Commun 4:1409.

183. Youngquist J. Tyler, Lennen Rebecca M., Ranatunga Don R., Bothfeld William H., II Wesley D. Marner, Pfleger Brian F. 2012. Kinetic modeling of free fatty acid production in Escherichia coli based on continuous cultivation of a plasmid free strain. Biotechnol Bioeng 109:1518–1527.

184. Elkins CA, Nikaido H. 2002. Substrate specificity of the RND-type multidrug efflux pumps AcrB and AcrD of *Escherichia coli* Is determined predominately by two large periplasmic loops. J Bacteriol 184:6490–6498.

185. Tikhonova EB, Zgurskaya HI. 2004. AcrA, AcrB, and TolC of *Escherichia coli* Form a Stable Intermembrane Multidrug Efflux Complex. J Biol Chem 279:32116–32124.

186. Du D, Wang Z, James NR, Voss JE, Klimont E, Ohene-Agyei T, Venter H, Chiu W, Luisi BF. 2014. Structure of the AcrAB–TolC multidrug efflux pump. Nature 509:512–515.

187. Mitschke J, Georg J, Scholz I, Sharma CM, Dienst D, Bantscheff J, Voß B, Steglich C, Wilde A, Vogel J, Hess WR. 2011. An experimentally anchored map of

transcriptional start sites in the model cyanobacterium Synechocystis sp. PCC6803. Proc Natl Acad Sci 108:2124–2129.

188. Opperman TJ, Kwasny SM, Kim H-S, Nguyen ST, Houseweart C, D'Souza S, Walker GC, Peet NP, Nikaido H, Bowlin TL. 2014. Characterization of a Novel Pyranopyridine Inhibitor of the AcrAB Efflux Pump of Escherichia coli. Antimicrob Agents Chemother 58:722–733.

189. Yuan W, Wiehn M, Wang Y, Kim HW, Rittmann BE, Nielsen DR. 2013. Solid-phase extraction of long-chain fatty acids from aqueous solution. Sep Purif Technol 106:1–7.

190. Castets A-M, Houmard J, Tandeau de Marsac N. 1986. Is cell motility a plasmid-encoded function in the cyanobacterium Synechocystis 6803? FEMS Microbiol Lett 37:277–281.

191. Kaneko T, Nakamura Y, Sasamoto S, Watanabe A, Kohara M, Matsumoto M, Shimpo S, Yamada M, Tabata S. 2003. Structural analysis of four large plasmids harboring in a unicellular cyanobacterium, *Synechocystis* sp PCC 6803. DNA Res 10:221–228.

192. Kotani H. 1994. A Physical Map of the Genome of a Unicellular Cyanobacterium Synechocystis sp. Strain PCC6803. DNA Res 1:303–307.

193. Kontani H, Tanaka A, Kaneko T, Sato S, Sugiura M, Tabata S. Assignment of 82 Known Genes and Gene Clusters on the Genome of the Unicellular Cyanobacteriurn *Synechocystis* sp Strain PCC6803 2:10.

194. Yang X, McFadden BA. 1993. A small plasmid, pCA2. 4, from the cyanobacterium Synechocystis sp. strain PCC 6803 encodes a rep protein and replicates by a rolling circle mechanism. J Bacteriol 175:3981–3991.

195. Gerlach MJ, Engelhart SE, Kemp AC, Moyer RP, Smoak JM, Bernhardt CE, Cahill N. 2017. Reconstructing Common Era relative sea-level change on the Gulf Coast of Florida. Mar Geol 390:254–269.

196. Mohapatra S, Mishra C, Behera SS, Thatoi H. 2017. Application of pretreatment, fermentation and molecular techniques for enhancing bioethanol production from grass biomass – A review. Renew Sustain Energy Rev 78:1007–1032.

197. Mian A, Sufi A. 2012. The Effects of Fiscal Stimulus: Evidence from the 2009 Cash for Clunkers Program*. Q J Econ 127:1107–1142.

198. Howarth RW, Ingraffea A, Engelder T. 2011. Should fracking stop? Nature 477:271.

199. Dal Bosco A, Gerencsér Z, Szendrő Z, Mugnai C, Cullere M, Kovàcs M, Ruggeri S, Mattioli S, Castellini C, Dalle Zotte A. 2014. Effect of dietary supplementation of Spirulina (Arthrospira platensis) and Thyme (Thymus vulgaris) on rabbit meat appearance, oxidative stability and fatty acid profile during retail display. Meat Sci 96:114–119.

200. Rashash DMC, Dietrich AM, Hoehn RC, Parker BC. 1995. The influence of growth conditions on odor-compound production by two chrysophytes and two cyanobacteria. Water Sci Technol 31:165–172.

201. Dietrich D, Hoeger S. 2005. Guidance values for microcystins in water and cyanobacterial supplement products (blue-green algal supplements): a reasonable or misguided approach? Toxicol Appl Pharmacol 203:273–289.

202. Swanson D, Block R, Mousa SA. 2012. Omega-3 Fatty Acids EPA and DHA: Health Benefits Throughout Life. Adv Nutr 3:1–7.

APPENDIX A

ADDITIONAL FIGURES

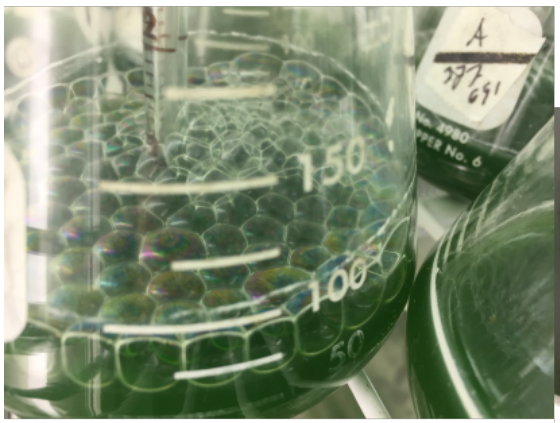


Figure A.1 Hydrocarbon Sheen (A) as shown in SD277 p*acrA* p*tolC*. It was also visible in SD277 p*acrA* p*acrB*, SD277 p*acrB*, and SD277 p*tolC* But it was not visible in other strains created in this research (B).

Figure A.2 Examples of plasmids used in this study with an example of the vector used to delete genes (A) and an example of the vectors used to add *acrB* (B)