UNIVERSITY OF CALIFORNIA, SAN DIEGO

Prophage Controls Biofilm Formation and Photosynthetic Pigmentation in the Cyanobacterium *Synechococcus elongatus* PCC 7942

A Thesis submitted in partial satisfaction of the requirements for the degree of Master of Science

in

Biology

by

Jingtong Wang

Committee in charge:

Professor Susan Golden, Chair
Professor Brian Palenik
Professor Elina Zuniga

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The Thesis of Jingtong Wang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

Prophage Controls Biofilm Formation and Photosynthetic Pigmentation in the Cyanobacterium *Synechococcus elongatus* PCC 7942

by

Jingtong Wang

Master of Science in Biology

University of California, San Diego, 2016

Professor Susan Golden, Chair

We have discovered a prophage present in the unicellular model cyanobacterium *Synechococcus elongatus* PCC 7942 that represses biofilm formation and photosynthetic pigmentation under high light conditions. While previously identified as a small cryptic phage region, the 49 kb prophage containing over 51 open reading frames in fact encodes a complete phage genome including all genes necessary for generating a virion structure, switching between lysogeny and lysis, and replication and packaging of its genome. We have genetically deleted the prophage from the *Synechococcus* genome, generating a phage-less strain. Unlike the lysogenic strain, which remains planktonic in culture and bleaches under high light, the phage-less strain readily forms biofilms and does not bleach. We hypothesize that the non-lysogenic strain of *Synechococcus* naturally grows as a biofilm at the bottom of the water column, where lower light conditions would favor darker pigmentation for increased photon capture, and that the prophage actively releases cells from the biofilm into higher light conditions near the top of the water column in order to subsequently enable ecological dispersal of the phage.

Chapter 1

Background

1.1 Statement of Purpose

The purpose of this project is to reveal how the distinction of a cyanobacterium of whether being in a suspended, single-cell state (planktonic) or a multicellular matrix (biofilm) on a surface is influenced by an integrated virus (prophage) into the cyanobacterial genome. This difference is important, because cyanobacterial biofilms in aquatic environments have both negative consequences when they form undesirably on surfaces, cause biofouling and produce biotoxins and positive consequences when they are applied in water treatment. Moreover, cyanobacteria are being developed as production organisms for bioproducts. The ability to regulate biofilm formation has applications for protecting and harvesting cyanobacterial crops due to the resistant to unwanted environmental stress and the natural capability of concentrating biomass.

1.2 Cyanobacteria: Widespread and Diverse Phototrophs

Cyanobacteria, also known as blue-green algae, are photosynthetic prokaryotes that can convert light into biologically useful forms of energy. These bacteria are among the most widespread and diverse microorganisms on the planet. Cyanobacteria occupy nearly every environment on the planet,

including freshwater, marine, Antarctic, desert, and endolithic ecosystems [1]. In aquatic environments, blooms of cyanobacteria can reach sizes that are visible by satellite [2]. These photosynthetic organisms can exist in planktonic forms, in phototrophic biofilm communities, or as endosymbionts in eukaryotes [1, 3].

The cyanobacteria are structurally and genetically diverse [4]. Populations range from unicellular to filamentous, with more complex structures such as sheets or hollow balls being formed by some species. Individual cells in the population may develop into specific cell types, including nitrogen-fixing heterocysts and spore-like akinetes [1].

1.2.1 Generating an Oxygen Atmosphere Through Photosynthesis

The oldest evidence for cellular organisms dates to approximately 3.5 billion years ago, where the Earth's atmosphere lacked oxygen and contained high amounts of methane and carbon dioxide. The microbes of the time likely survived as chemolithotrophs utilizing anaerobic metabolic processes [5]. The emergence of photolithotrophs in the form of the cyanobacteria is believed to have occurred approximately 2.7 billion years ago. The subsequent evolution and success of oxygenic photosynthesis in cyanobacteria led to a momentous geographic event known as the great oxidation event (GOE) approximately 2.5 billion years ago that changed the early anoxic environment of Earth, resulting in the presence of free oxygen in the oceans and the atmosphere [5, 6]. This in turn led to increased bioavailability of nutrients through oxidation-dependent

solubilization and the evolution of aerobic respiration [6]. Therefore, without the evolution of cyanobacteria, the key evolutionary steps towards the development of eukaryotes and multicellular organisms would likely not have occurred.

1.2.2 Cyanobacteria: Ancestors of the Chloroplast

Cyanobacterial photosynthesis did not just provide oxygen flux to aerobic respiration. Free-living cyanobacteria are also responsible for the evolution of algae and plants. As a result of at least one endosymbiotic event, cyanobacteria supplied the products of photosynthesis to the host eukaryote and eventually integrated with the symbiotic host, thereby becoming the key organelle of modern photosynthetic eukaryotes known as the chloroplast [7]. Therefore, the evolution of cyanobacteria is ultimately responsible for all plant-type photosynthesis and free oxygen on the planet.

1.2.3 Impact of Cyanobacteria Today

Cyanobacteria still have a tremendous impact on today's ecosystem. Primary production of fixed carbon in organic compounds is mainly driven by oxygenic photosynthesis [8]. Cyanobacteria in both marine and terrestrial ecosystems contribute significantly to the global primary production [8]. Approximately 60% to 70% of CO_2 fixation in marine water systems is performed by two cyanobacterial clades: *Synechococcus* and *Prochlorococcus* [9].

Other cyanobacterial species, such as *Trichodesmium* [10, 11], are the main sources of global bioavailable nitrogen due to their ability to fix atmospheric nitrogen into ammonia and extensive prevalence in the ecosystem [12]. It has been estimated that *Trichodesmium* alone accounts for approximately 40% of global nitrogen fixation [13]. Therefore, cyanobacteria are the primary contributors to global carbon and nitrogen cycles, thus providing nutrients and energy to the global ecosystem.

Cyanobacteria directly impact human health and nutrition. A number of cyanobacteria have been approved for human and animal consumption, including *Aphanizomenon flos-aquae* and *Arthrospira platensis*, often called Spirulina. These cyanobacteria, consumed as either whole foods or as nutritional supplements, are high quality sources of all essential amino acids, polyunsaturated fatty acids including omega-3 fatty acids like Eicosapentaenoic acid (EPA), vitamins, minerals, and anti-oxidants [14, 15]. Consumption of cyanobacteria has also been shown to have anti-oxidative, anti-inflammatory, and anti-cancer properties [16]. Given the potential relative costs, land use requirements, and yields involved in mass-producing cyanobacteria as compared to livestock and other terrestrial agricultural sources of protein, consumption of cyanobacteria as a primary food source has been proposed as a solution to increased global food (particularly protein) demands.

In contrast to the nutritional benefits of some cyanobacterial species, a number of cyanobacterial species produce and secrete cyanotoxins, which

include potent neurotoxins, hepatotoxins, dermatoxins, cytotoxins, and endotoxins [17]. Blooms of these toxin-producing species, often called harmful algal blooms (HABs) can produce toxins in high enough concentrations to poison animals and humans via exposure or ingestion of the infected water. Alternatively, cyanotoxins can accumulate in animals over time, which can result in poisoning if the animal is eaten as in the case with shellfish poisoning. With increasing global temperatures, the frequency and scale of harmful algal blooms is increasing [18-20]. For example, algal blooms dominated by *Microcystis aeruginosa*, a cyanobacteria capable of producing the hepatotoxin microcystin, have occurred annually over the past eight years [21, 22]. These blooms reached peaks sizes of approximately 5,000 km^2 and resulted in temporary shut downs of drinking water supplies to Carroll Township, Ohio in 2013 and Toledo, Ohio in 2014 due to high concentrations of microcystin [23]. Monitoring and controlling these blooms is a critical concern to environmental and human health agencies.

1.2.4 Biotechnological Applications of Cyanobacteria

Cyanobacteria are an attractive cellular factory or synthetic biology chassis for producing commodity chemicals and renewable fuels [24]. First, a number of genetically manipulable cyanobacterial systems have been established, including the unicellular obligate photoautotroph *Synechococcus elongatus* PCC 7942 [25], the filamentous and nitrogen-fixing *Anabaena* sp. PCC 7120 [26], the unicellular heterotrophic *Synechocystis* sp. PCC 6803 [27],

and the filamentous model production strain *Leptolyngbya* sp. BL0902 [28]. Second, the ability to utilize solar energy to empower carbon fixation and subsequent metabolism makes cyanobacteria a sustainable alternative to other genetically modifiable organisms, such as *E. coli*, because they do not need to be provided with a chemical energy source such as carbohydrates [29]. In some of these organisms, metabolic engineering has already yielded the ability to produce a number of desired products, including ethanol, lactic acid, sucrose, ethylene, isoprene, iso-butyraldehyde, iso-butanol, and 1-butanol [30].

The elimination of carbohydrate feeds not only reduces production costs, but also enables net zero carbon emission through fixation of CO_2. Compared to current biofuel production methods, such as the generation of cellulosic ethanol, cyanobacteria can produce higher yields from solar energy with less water and less arable land. Because some cyanobacterial strains are capable of using salt water, brackish water, or polluted waste water for growth, cyanobacterial production systems may not need to compete with human consumption for potable water sources [31]. These additional advantages over current practices make cyanobacteria a promising biotechnological platform.

1.3 Bacteriophage

Like other bacteria, cyanobacteria are hosts to variety of viruses. A bacteriophage, or simply phage, is a virus that infects and replicates within a bacterium. Phage have been the subject of modern biological research for over 100 years. This research, often at the forefront of scientific and technological

innovations, has revealed much about the structures, life cycles, and molecular mechanisms enabling the replication of these viruses. A resurgence of interest in phage research has occurred in recent years: there is a greater appreciation for the prevalence, diversity, and impact of phage in all environments; phage are a source of promising technologies that will affect biomedical, energy, electronics, and material science applications, among others; and renewed investigations into phage therapy hold the potential for a solution to the antibiotic resistance crisis [32, 33]. Although a comprehensive review of the phage literature is beyond the scope of this thesis, topics critical to the results of this project are discussed below.

1.3.1 Phage Life Cycles

Phage replication occurs inside of the host cell, taking advantage of the host's cellular machinery and molecular resources, either through a lytic cycle or a lysogenic cycle (**Figure 1- 1**). Virulent phages propagate solely through the lytic cycle, where the phage hijacks the host's replication, transcription, and translational components in order to generate new copies of the viral genome along with the structural components of the virion particle. Often, this is concomitant with destruction of the host genome and proteins in order to generate more building blocks for phage replication. Lysis of the cell releases assembled virion particles into the environment, where further rounds of host recognition and infection can occur.

Temperate phages are capable of propagating through the lysogenic

cycle, where the phage genome integrates into the host genome and is replicated along with the host's DNA. In the lysogenic state, the integrated phage is called a prophage and the host is a lysogen. Environmental stresses or the alterations to the metabolic state of the cell can stimulate induction of the prophage through a genetically-encoded switch that stops lysogenic maintenance and initiates the lytic cycle through excision of the phage. Over time, a prophage that remains latent may accumulate mutations that result in the disabling of the phage's ability to induce, yielding a permanently latent or cryptic prophage remnant in the host genome.

Although the lysogenic state is generally a dormant state, it can be evolutionarily advantageous to the host cell. The phage may carry genes acquired from a previous host that can prove beneficial to the lysogen, a process known as horizontal gene transfer. In some cases, lysogenic conversion can occur when integration of the prophage disrupts expression of specific genes or prophage-encoded genes are expressed in the lysogen and confer new properties to the bacterium. The most common examples of lysogenic conversion include superinfection immunity from subsequent infections by other phage, modifications to the bacterial O-antigen, and virulence through phage-encoded toxins. In some cases, lysogenic conversion can impact more complex traits such as biofilm formation and sporulation [34]. In the case of biofilm formation, it has been demonstrated that cryptic prophage elements in the *E. coli* K12 strain [35] and expression of an RNA polymerase sigma factor from lysogenized phage genomes in *Bacillus anthracis* enable

biofilm formation [36]. Given that biofilm formation can enable antibiotic resistance and determine population dispersal kinetics, this kind of lysogenic conversion can have tremendous impacts on human health and the environment.

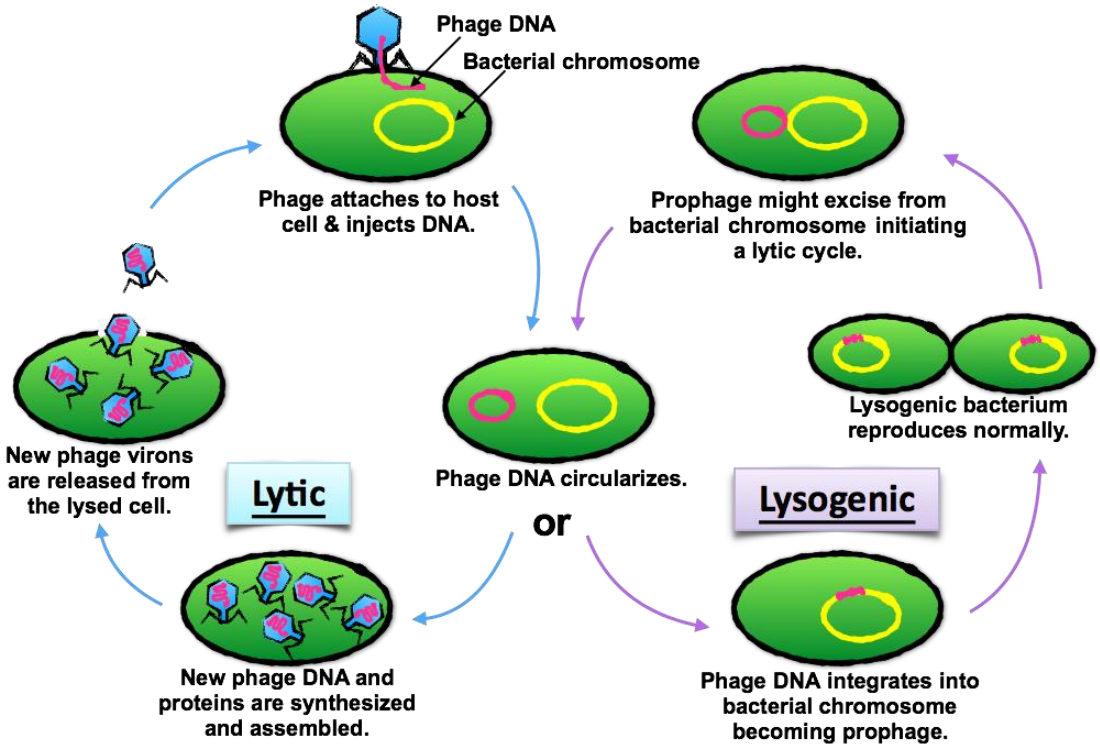

Figure 1- 1. The lytic and lysogenic cycles of a phage. Adapted from Pearson Education Benjamin Cummings.

1.3.2 Cyanophage

Phages that specifically infect cyanobacteria are called cyanophage. Until approximately a decade ago, very little was known about cyanophage especially in relation to the body of knowledge concerning enteric phage. Cyanophage have been predominantly studied in marine environments where it is thought that they have a major impact on the planet's primary production of fixed carbon through lytic modulation of oceanic cyanobacterial population dynamics [37-39]. Like the enteric phage, cyanphage can replicate through lysis or lysogeny [40]. Thus, they are also a major mechanism for horizontal gene transfer [41]. For example, multiple cyanophage families that infect marine *Prochlorococcus* species carry genes encoding the photosystem II core reaction center protein D1 (*psbA*), and a high-light-inducible protein (HLIP) (*hli*), along with other *hli* and photosystem genes [42]. These genes are believed to provide a fitness advantage to these virulent phages during the lytic cycle by maintaining the photosynthetic apparatus in the absence of the host's genome in order to provide more energy and resources for phage replication.

In contrast to the marine systems, freshwater cyanobacteria have not been as well studied. As of a 2013 review, only 6 freshwater cyanophage genomes have been sequenced, whereas >200 marine cyanophage genomes have been sequenced and numerous metagenomes have been published [43]. Only a handful of freshwater cyanophage have been sequenced in the last decade [44-46]. While the environmental impacts of freshwater cyanophage are presumed to be similar to their marine counterparts, this has not been well

studied [44]. Only recently has it been demonstrated that freshwater cyanophage can also carry genes encoding host proteins, such as NblA, which degrades the light-harvesting phycobilisomes [47, 48].

It is believed that phage-encoded host genes enhance lytic reproduction. In contrast, the lysogenic state is predominantly viewed as hibernetic, with little or no contribution of phage genes to the biology of the host (**Figure 1- 2**). However, this hypothesis has been proposed based primarily on sequence similarity and a single report of functional analysis through heterologous expression of the phage-encoded NblA protein in a non-host cyanobacterium [48, 49]. The main difficulty in testing the impacts of cyanophage-encoded genes on their hosts is that there are NO known genetic systems in which both the phage and cyanobacterium can be easily manipulated in tandem. Thus, without further investigations, the belief that the prophage does not impact the host's behavior or physiology in a manner similar to that observed in the lytic stage has prevailed [43] and temperate cyanophage are viewed as merely agents of horizontal gene transfer [48].

In this thesis, I will show that a freshwater cyanophage can control its host's niche through alterations in biofilm behavior and photosynthetic metabolism during the lysogenic cycle. These findings will allow us to not only better understand the impacts of freshwater cyanophage, but will also enable us to develop phage and genetic tools in order to improve biofuel production through improved harvesting and efficient energy conversion, as well as manipulate toxic cyanobacterial populations.

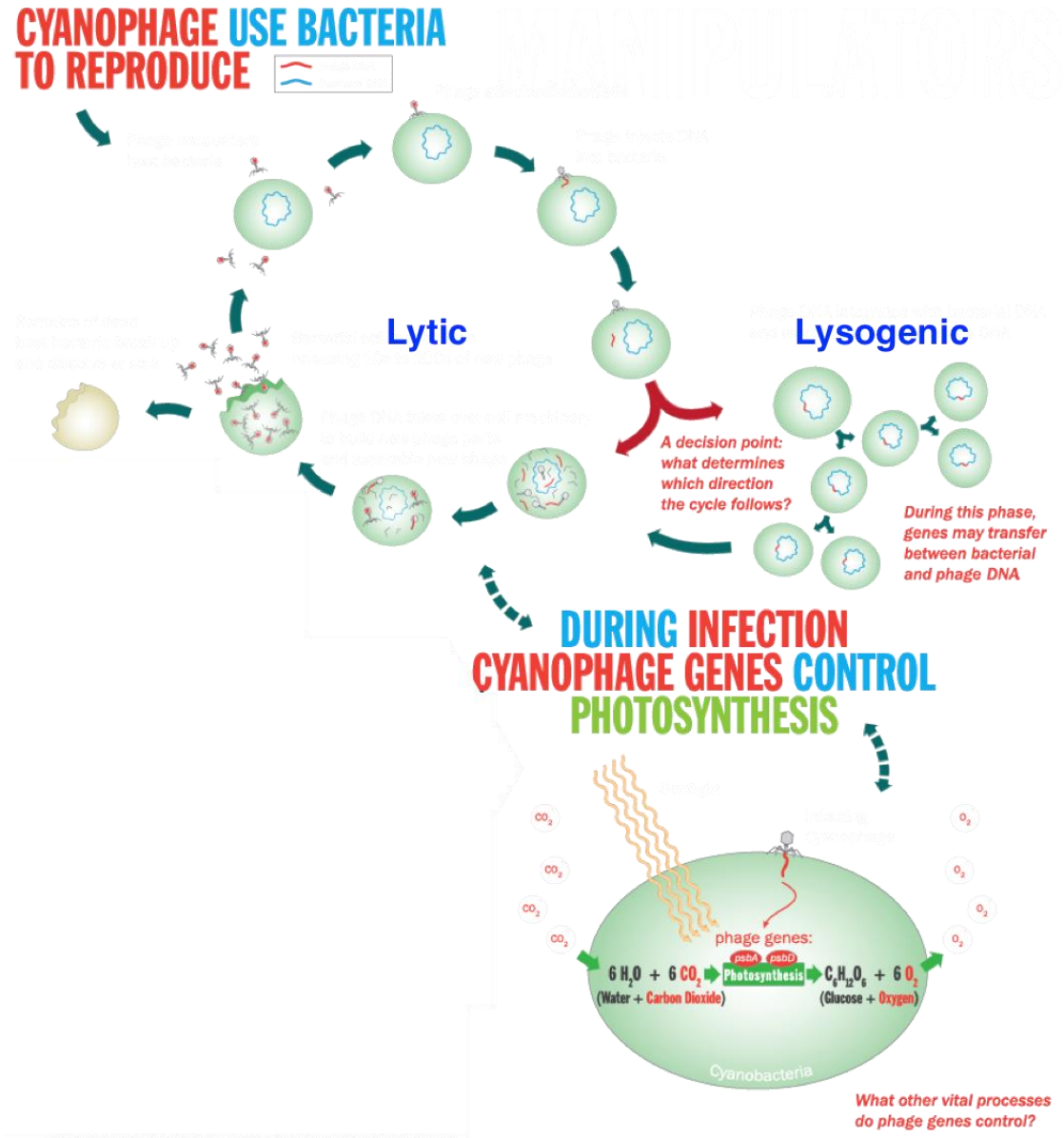

Figure 1- 2. The lytic and lysogenic cycle of the cyanophage. During infection, cyanophage genes *psbA* and *psbD* control photosynthesis that is difficult to test genetically. Adapted from Matthew Sullivan, 2015.

1.4 Reference

1.Whitton, B.A. and M. Potts. *The ecology of cyanobacteria: their diversity in time and space*, edited. Dordrecht ; Boston: Kluwer Academic. 2000

2.Kahru, M. "Quasi-true color images of the Baltic - looking for cyanobacteria blooms." Last modified 2012. http://spg.ucsd.edu/Satellite_Projects/BalticCyano2012/BalticCyano2012.htm

3.Raven, J. and J. Allen. "Genomics and chloroplast evolution: what did cyanobacteria do for plants?" *Genome biology* 4, no. 3 (2003): 209. doi: 10.1186/gb-2003-4-3-209

4.Hess, W.R. "Cyanobacterial genomics for ecology and biotechnology." *Curr Opin Microbiol* 14, no. 5 (2011): 608-614. doi: 10.1016/j.mib.2011.07.024

5.Schopf, J.W. "Geological evidence of oxygenic photosynthesis and the biotic response to the 2400-2200 ma "great oxidation event"." *Biochemistry. Biokhimiia* 79, no. 3 (2014): 165-177. doi: 10.1134/S0006297914030018

6.Knoll, A.H. "Paleobiological Perspectives on Early Microbial Evolution." *Cold Spring Harbor perspectives in biology* 7, no. 7 (2015): a018093. doi: 10.1101/cshperspect.a018093

7.Thompson, A.W., R.A. Foster, A. Krupke, B.J. Carter, N. Musat, D. Vaulot, M.M.M. Kuypers, and J.P. Zehr. "Unicellular cyanobacterium symbiotic with a single-celled eukaryotic alga." *Science (New York, N.Y.)* 337, no. 6101 (2012): 1546-1550. doi: 10.1126/science.1222700

8.Field, C.B., M.J. Behrenfeld, J.T. Randerson, and P. Falkowski. "Primary production of the biosphere: integrating terrestrial and oceanic components." *Science (New York, N.Y.)* 281, no. 5374 (1998): 237-240

9.Partensky, F., W.R. Hess, and D. Vaulot. "Prochlorococcus, a marine photosynthetic prokaryote of global significance." *Microbiology and molecular biology reviews : MMBR* 63, no. 1 (1999): 106-127

10.Capone, D.G., J.P. Zehr, H.W. Paerl, B. Bergman, and E.J. Carpenter. "Trichodesmium, a Globally Significant Marine Cyanobacterium." *Science (New York, N.Y.)* 276, no. 5316 (1997): 1221-1229. doi: 10.1126/science.276.5316.1221

11.Bergman, B., G. Sandh, S. Lin, J. Larsson, and E.J. Carpenter. "Trichodesmium – a widespread marine cyanobacterium with unusual nitrogen fixation properties." *FEMS microbiology reviews* 37, no. 3 (2013): 286-302. doi: 10.1111/j.1574-6976.2012.00352.x

12.Flores, E. and A. Herrero. "Nitrogen assimilation and nitrogen control in cyanobacteria." *Biochemical Society transactions* 33, no. 1 (2005): 164-167. doi: 10.1042/BST0330164

13.Berman-Frank, I., P. Lundgren, and P. Falkowski. "Nitrogen fixation and photosynthetic oxygen evolution in cyanobacteria." *Research in microbiology* 154, no. 3 (2003): 157-164. doi: 10.1016/S0923-2508(03)00029-9

14.Belay, A., Y. Ota, K. Miyakawa, and H. Shimamatsu. "Current knowledge on potential health benefits of Spirulina." *Journal of Applied Phycology* 5, no. 2 (1993): 235-241. doi: 10.1007/BF00004024

15.Belay, A. "The Potential Application of Spirulina (Arthrospira) as a Nutritional and Therapeutic Supplement in Health Management." *The Journal of the American Nutraceutical Association* 5, no. 2 (2002)

16.Lee, J.-C., M.-F. Hou, H.-W. Huang, F.-R. Chang, C.-C. Yeh, J.-Y. Tang, and H.-W. Chang. "Marine algal natural products with anti-oxidative, anti-inflammatory, and anti-cancer properties." *Cancer Cell International* 13, no. 1 (2013): 1-7. doi: 10.1186/1475-2867-13-55

17.Mankiewicz, J., M. Tarczynska, Z. Walter, and M. Zalewski. "Natural toxins from cyanobacteria." *Acta Biologica Cracoviensia Series Botanica* 45, no. 2 (2003): 2-9

18.Stumpf, R.P., T.T. Wynne, D.B. Baker, and G.L. Fahnenstiel. "Interannual Variability of Cyanobacterial Blooms in Lake Erie." *PloS one* 7, no. 8 (2012): e42444. doi: 10.1371/journal.pone.0042444

19.Paerl, H.W. and J. Huisman. "Blooms Like It Hot." *Science (New York, N.Y.)* 320, no. 5872 (2008): 57-58. doi: 10.1126/science.1155398

20.Verspagen, J.M.H., D.B. Van de Waal, J.F. Finke, P.M. Visser, E. Van Donk, and J. Huisman. "Rising CO₂ Levels Will Intensify Phytoplankton Blooms in Eutrophic and Hypertrophic Lakes." *PloS one* 9, no. 8 (2014): e104325. doi: 10.1371/journal.pone.0104325

21.Michalak, A.M., E.J. Anderson, D. Beletsky, S. Boland, N.S. Bosch, T.B. Bridgeman, J.D. Chaffin, K. Cho, R. Confesor, I. Daloğlu, J.V. DePinto, M.A. Evans, G.L. Fahnenstiel, L. He, J.C. Ho, L. Jenkins, T.H. Johengen, K.C. Kuo, E. LaPorte, X. Liu, M.R. McWilliams, M.R. Moore, D.J. Posselt, R.P. Richards, D. Scavia, A.L. Steiner, E. Verhamme, D.M. Wright, and M.A. Zagorski. "Record-setting algal bloom in Lake Erie caused by agricultural and meteorological trends consistent with expected future conditions." *Proceedings of the National Academy of Sciences* 110, no. 16 (2013): 6448-6452

22.Wynne, T. and R. Stumpf. "Spatial and Temporal Patterns in the Seasonal Distribution of Toxic Cyanobacteria in Western Lake Erie from 2002–2014." *Toxins* 7, no. 5 (2015): 1649

23.Yeager-Kozacek, C. "Great Lakes Drinking Water Fouled by Toxic Algae." Last modified 2014. http://www.circleofblue.org/waternews/2014/world/choke-point-index-great-lakes-drinking-water-fouled-by-toxic-algae/

24.Wang, B., J. Wang, W. Zhang, and D.R. Meldrum. "Application of synthetic biology in cyanobacteria and algae." *Frontiers in microbiology* 3, (2012): 344. doi: 10.3389/fmicb.2012.00344

25.Golden, S.S., J. Brusslan, and R. Haselkorn. "Genetic engineering of the cyanobacterial chromosome." *Methods in enzymology* 153, (1987): 215-231

26.Wolk, C.P., A. Vonshak, P. Kehoe, and J. Elhai. "Construction of shuttle vectors capable of conjugative transfer from Escherichia coli to nitrogen-fixing filamentous cyanobacteria." *Proceedings of the National Academy of Sciences of the United States of America* 81, no. 5 (1984): 1561-1565

27.Chauvat, F., L. Vries, A. Ende, and G.A. Arkel. "A host-vector system for gene cloning in the cyanobacterium Synechocystis PCC 6803." *Molecular and General Genetics* 204, no. 1 (1986): 185-191. doi: 10.1007/BF00330208

28.Taton, A., E. Lis, D.M. Adin, G. Dong, S. Cookson, S.A. Kay, S.S. Golden, and J.W. Golden. "Gene Transfer in *Leptolyngbya sp.* Strain BL0902, a Cyanobacterium Suitable for Production of Biomass and Bioproducts." *PloS one* 7, no. 1 (2012): e30901. doi: 10.1371/journal.pone.0030901

29.Huang, H.H., D. Camsund, P. Lindblad, and T. Heidorn. "Design and characterization of molecular tools for a Synthetic Biology approach towards developing cyanobacterial biotechnology." *Nucleic Acids Research* 38, no. 8 (2010): 2577-2593. doi: 10.1093/nar/gkq164

30.Wijffels, R.H., O. Kruse, and K.J. Hellingwerf. "Potential of industrial biotechnology with cyanobacteria and eukaryotic microalgae." *Current opinion in biotechnology* 24, no. 3 (2013): 405-413. doi: 10.1016/j.copbio.2013.04.004

31.Pade, N. and M. Hagemann. "Salt acclimation of cyanobacteria and their application in biotechnology." *Life (Basel, Switzerland)* 5, no. 1 (2014): 25-49. doi: 10.3390/life5010025

32.Ventola, C.L. "The Antibiotic Resistance Crisis: Part 1: Causes and Threats." *Pharmacy and Therapeutics* 40, no. 4 (2015): 277-283

33.Kutter, E.M., S.J. Kuhl, and S.T. Abedon. "Re-establishing a place for phage therapy in western medicine." *Future microbiology* 10, no. 5 (2015): 685-688. doi: 10.2217/fmb.15.28

34.Fortier, L.-C. and O. Sekulovic. "Importance of prophages to evolution and virulence of bacterial pathogens." *Virulence* 4, no. 5 (2013): 354-365. doi: 10.4161/viru.24498

35.Wang, X., Y. Kim, Q. Ma, S.H. Hong, K. Pokusaeva, J.M. Sturino, and T.K. Wood. "Cryptic prophages help bacteria cope with adverse environments." *Nature communications* 1, (2010): 147. doi: 10.1038/ncomms1146

36.Schuch, R. and V.A. Fischetti. "The Secret Life of the Anthrax Agent <italic>Bacillus anthracis</italic>: Bacteriophage-Mediated Ecological Adaptations." *PloS one* 4, no. 8 (2009): e6532. doi: 10.1371/journal.pone.0006532

37.Mann, N.H., A. Cook, A. Millard, S. Bailey, and M. Clokie. "Marine ecosystems: bacterial photosynthesis genes in a virus." *Nature* 424, no. 6950 (2003): 741-741. doi: 10.1038/424741a

38.Koskella, B. and M.A. Brockhurst. "Bacteria-phage coevolution as a driver of ecological and evolutionary processes in microbial communities." *FEMS microbiology reviews* 38, no. 5 (2014): 916-931. doi: 10.1111/1574-6976.12072

39.Lindell, D., J.D. Jaffe, Z.I. Johnson, G.M. Church, and S.W. Chisholm. "Photosynthesis genes in marine viruses yield proteins during host infection." *Nature* 438, no. 7064 (2005): 86-89. doi: 10.1038/nature04111

40.Jiang, S.C. and J.H. Paul. "Significance of Lysogeny in the Marine Environment: Studies with Isolates and a Model of Lysogenic Phage Production." *Microbial Ecology* 35, no. 3: 235-243. doi: 10.1007/s002489900079

41.Ignacio-Espinoza, J.C. and M.B. Sullivan. "Phylogenomics of T4 cyanophages: lateral gene transfer in the 'core' and origins of host genes." *Environmental Microbiology* 14, no. 8 (2012): 2113-2126. doi: 10.1111/j.1462-2920.2012.02704.x

42.Lindell, D., M.B. Sullivan, Z.I. Johnson, A.C. Tolonen, F. Rohwer, and S.W. Chisholm. "Transfer of photosynthesis genes to and from Prochlorococcus viruses." *Proceedings of the National Academy of Sciences of the United States of America* 101, no. 30 (2004): 11013-11018. doi: 10.1073/pnas.0401526101

43.Xia, H., T. Li, F. Deng, and Z. Hu. "Freshwater cyanophages." *Virologica Sinica* 28, no. 5 (2013): 253-259. doi: 10.1007/s12250-013-3370-1

44.Dreher, T.W., N. Brown, C.S. Bozarth, A.D. Schwartz, E. Riscoe, C. Thrash,

S.E. Bennett, S.-C. Tzeng, and C.S. Maier. "A freshwater cyanophage whose genome indicates close relationships to photosynthetic marine cyanomyophages." *Environmental Microbiology* 13, no. 7 (2011): 1858-1874. doi: 10.1111/j.1462-2920.2011.02502.x

45.Sullivan, M.B., B. Krastins, J.L. Hughes, L. Kelly, M. Chase, D. Sarracino, and S.W. Chisholm. "The genome and structural proteome of an ocean siphovirus: a new window into the cyanobacterial 'mobilome'." *Environmental Microbiology* 11, no. 11 (2009): 2935-2951. doi: 10.1111/j.1462-2920.2009.02081.x

46.Chénard, C., A.M. Chan, W.F. Vincent, and C.A. Suttle. "Polar freshwater cyanophage S-EIV1 represents a new widespread evolutionary lineage of phages." *The ISME journal* 9, no. 9 (2015): 2046-2058. doi: 10.1038/ismej.2015.24

47.Yoshida, T., K. Nagasaki, Y. Takashima, Y. Shirai, Y. Tomaru, Y. Takao, S. Sakamoto, S. Hiroishi, and H. Ogata. "Ma-LMM01 Infecting Toxic Microcystis aeruginosa Illuminates Diverse Cyanophage Genome Strategies." *Journal of Bacteriology* 190, no. 5 (2008): 1762-1772

48.Gao, E.B., J.F. Gui, and Q.Y. Zhang. "A Novel Cyanophage with a Cyanobacterial Nonbleaching Protein A Gene in the Genome." *Journal of Virology* 86, no. 1 (2011): 236-245. doi: 10.1128/JVI.06282-11

49.Ma, Y., L.Z. Allen, and B. Palenik. "Diversity and genome dynamics of marine cyanophages using metagenomic analyses." *Environmental microbiology reports* 6, no. 6 (2014): 583-594. doi: 10.1111/1758-2229.12160

Chapter 2

A Prophage in Synechococcus elongatus PCC 7942

2.1 Introduction to *Synechococcus elongatus* PCC 7942

Synechococcus elongatus PCC 7942 is a unicellular, polyploid cyanobacterium that is naturally competent [1], can be transformed with exogenous DNA [2], and can integrate DNA into or otherwise alter its chromosome through apparent double homologous recombination [3, 4]. Due to this ease of manipulation, *S. elongatus* is an excellent genetic model system that has been applied to study, among other subjects, circadian rhythms and metabolic engineering for the production of biofuels and high-value co-products [5-7]. Although the laboratory strain of *S. elongatus* is planktonic, it has recently been used to study the regulation and formation of biofilms due to the discovery that a knockout mutation in the type II secretion system gene E enables biofilm formation [8].

2.2 Discovery, description, and annotations

Through bioinformatics analysis, a postdoc in the Golden lab, Dr. Ryan Simkovsky, identified a complete prophage (bases 711,254 to 759,991) in the chromosome of *S. elongatus* PCC 7942 comprising 48,618 bp flanked by two identical 60 bp duplications. While others have noted that a cryptic prophage [9] or that a prophage-like 20 kb region [10] exists at this location in the

chromosome, Dr. Simkovsky's analysis indicates that the prophage encodes all the genes necessary to construct virion particles likely composed of an icosahedral head and a long P2-like tail; for excision, replication, and integration of the phage genome; and for regulation of a temperate lifecycle between lytic and lysogenic phases. Genes that encode similar function, such as the tail genes, are encoded in the same orientation and in groups, allowing the annotation of gene sections (). Analysis of the flanking 60 bp duplication indicates that this sequence is the phage's attachment site allowing the integration of the prophage into a tRNA-Leucine (tRNA-leu) gene without disrupting the gene (). In spite of this analysis, a number of hypothetical ORFs with no known homologs or function remain to be investigated.

As shown in the gene sections map of the prophage (), section 1 is composed of a single gene (Synpcc7942_0716) encoding the integrase; section 2 contains hypothetical genes, the putative DNA excisionase, and the dUTP synthetase; and section 3 contains homologs of phage genes responsible for DNA replication and packaging, such as the phage terminase. Sections 4 and 5 contain the structural genes encoding the head and tail, respectively, while section 6 is composed of a single hypothetical gene of unknown function. The phage lysozyme, hypothetical proteins, and putative DNA-binding proteins are encoded in section 7, while the genes in section 8 and 9 are responsible for maintaining lysogeny or inducing lysis, respectively.

Altered versions of the prophage are present in the genomes of the closely related strains of *Synechococcus elongatus* PCC 6301 [11] and

*Synechococcus elongatu*s UTEX 2973 [12]. In PCC 6301, the integration site and attachment site duplications are identical to PCC 7942; however, the prophage sequences differ by 39 single nucleotide polymorphisms (SNPs), 3 single base insertions, and 1 single base deletion as compared to PCC 7942 in the prophage. 32 of these differences are located in the tail gene section (section 5). The nucleotide sequence of the UTEX 2973 prophage region is identical to that of PCC 7942 except for the deletion of the terminal 220 bp of the prophage as part of a 5,764 bp deletion that includes 55 bp of the last prophage gene (Synpcc7942_0767), the entirety of the second attachment site, and five genes outside of the prophage (Synpcc7942_0768 to Synpcc7942_0772). In theory, the deletion of the duplicate attachment site in UTEX 2973 would render its prophage incapable of excision.

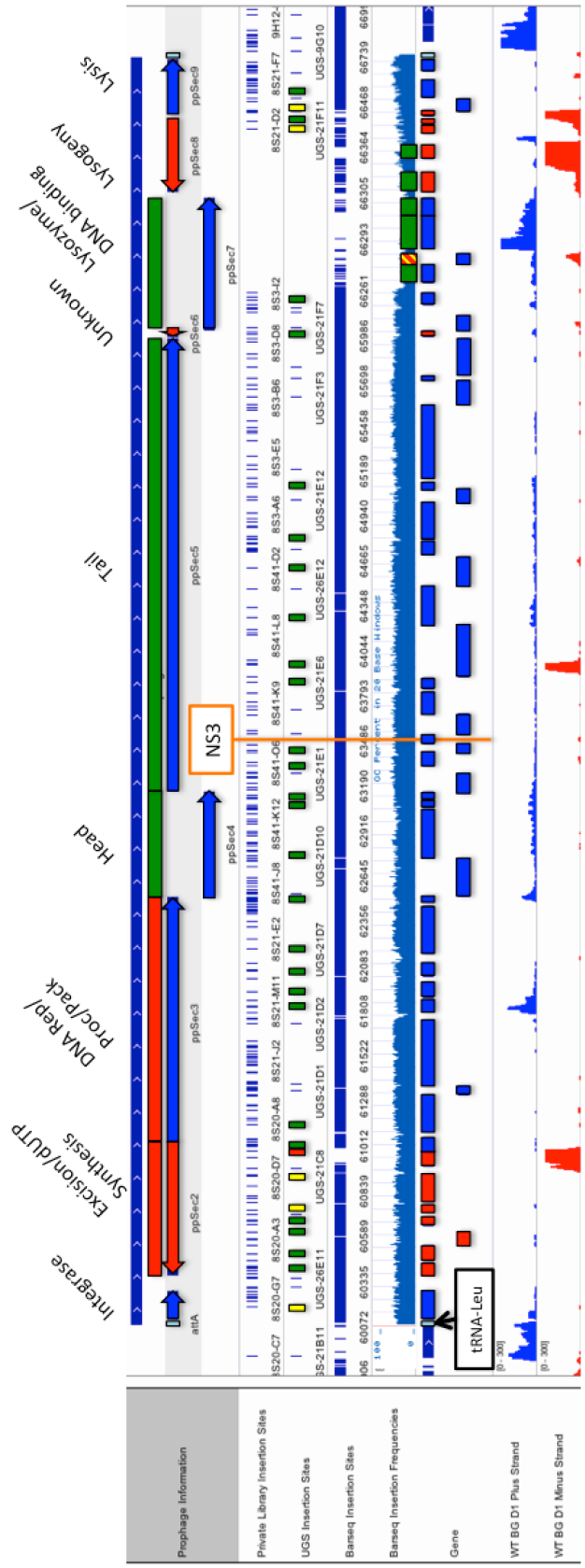

Figure 2- 1. The prediction of a complete prophage in *Synechococcus elongutas* PCC 7942 by Dr. Ryan Simkovsky.The prophage genes start from 711,254 to 759,991 (SynPCC7942_0716 – SynPCC7942_0767), which inserts into a tRNA-Leucine.

2.3 ∆50kb: A Strain of *S. elongatus* lacking the prophage

Serendipitously, at about the same time as Dr. Simkovsky was performing his analysis, the Yoshikawa lab in Japan published a study on the origin of replication of PCC 7942 that demonstrated that their lab's strain of PCC 7942 lacked the exact region encoding the prophage [13]. While their publication essentially ignored this deletion as it was inconsequential to their study, we recognized that their strain lacked the prophage. Because the precise history of this strain is unknown (personal communication, Yoshikawa), it is unclear whether the prophage excised or this strain represents a lineage in which the prophage never integrated. Reanalysis of the published sequencing data for this strain confirmed the absence of the prophage while demonstrating that SNPs are present in six open reading frames in the chromosome, as compared to the published genome sequence (**Table 2-1**). The most obviously significant of these mutations is a truncation mutation in the sigma factor gene, sigF (Synpcc7942_1510). The impacts of this sigma factor mutation will be discussed in Chapter 4. We obtained this strain, hereafter referred to as ∆50kb, and observed that ∆50kb settles and forms biofilms in stationary flask culture (**Figure 2- 2**), and is darker on solid surfaces grown under high light as compared to our lab strain (hereafter referred to as WT).

Table 2-1. All SNPs identified in *Synechococcus elongatus* PCC 7942 obtained from Yoshikawa Lab as compared to the published sequence for our lab WT.

Mutation	Gene
P87P (CCC→CCA)	*Synpcc7942_0778*
T206P (ACC→CCC)	*Synpcc7942_0807*
R768L (CGT→CTT)	*Synpcc7942_0859*
K53E (AAG→GAG)	*Synpcc7942_0977*
Q149* (CAA→TAA)	*Synpcc7942_1510*
V294A (GTG→GCG)	pyrG

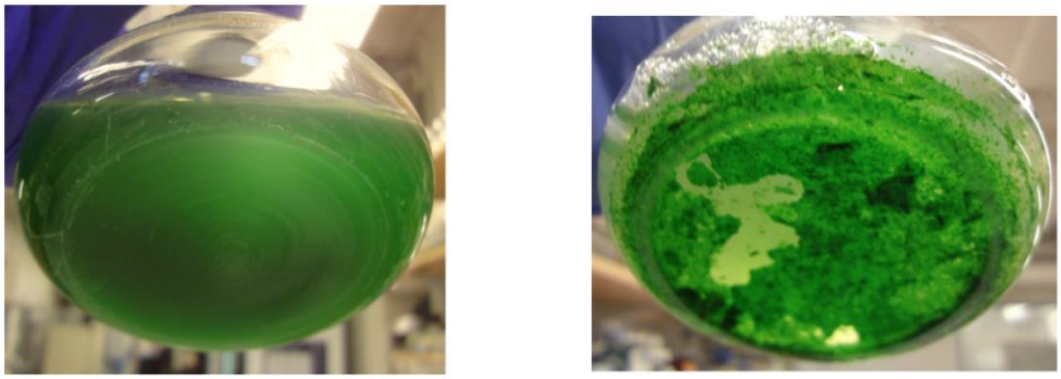

Figure 2- 2. Δ50kb, which lacks the prophage, settles and forms biofilms.Δ50kb was obtained from Yoshikawa Lab and was identified as having a 50-kbp deletion (711, 254 - 759,931) relative to the Golden lab WT. Δ50kb was confirmed as lacking the prophage region via PCR and genome sequencing analysis.

2.4 Hypothesis

Based upon the ∆50kb biofilm formation phenotype, we developed a model in which the prophage regulates the behavior and ecology of *S. elongatus* in natural environments. The goal of this project is to test this hypothesized model.

As previously stated, the Golden lab's WT PCC 7942 is a lysogen and remains planktonic in liquid cultures (**Figure 2- 3**) Our preliminary data suggested that when PCC 7942 lacks the prophage, it forms biofilms, which are surface-associated bacterial communities embedded within self-produced extracellular polymeric substances. In support of this proposal, our collaborators from Dr. Rakefet Schwarz's Lab in Israel have demonstrated that PCC 7942 encodes genetic mechanisms for regulating and producing biofilms [8]. Their work has demonstrated that the laboratory strain of PCC 7942 produces an extracellular repressor of biofilm formation, though the identity of this repressive molecule is currently unknown. In natural environments, settling and biofilm-forming cells would attach to surfaces at the bottom of the water column, where light levels are low. To harvest enough light to meet the cells' energy requirements, these cells have increased concentrations of photosynthetic pigments and appear darker in color. These biofilms are a target for phage infection. Upon infection and integration of the phage, the prophage represses the biofilm state so that *S. elongatus* becomes planktonic and rises in the water column. The prophage also represses the photosynthetic light-harvesting apparatus, resulting in a pale appearance, in order to better

acclimate the cell to the higher light intensities at the top of the water column. The prophage-induced planktonicity thus enables the cell to move horizontally through the environment. This in turn allows for phage migration to new environments where induction of lysis and dispersal of virions would allow for the infection of new cyanobacterial populations.

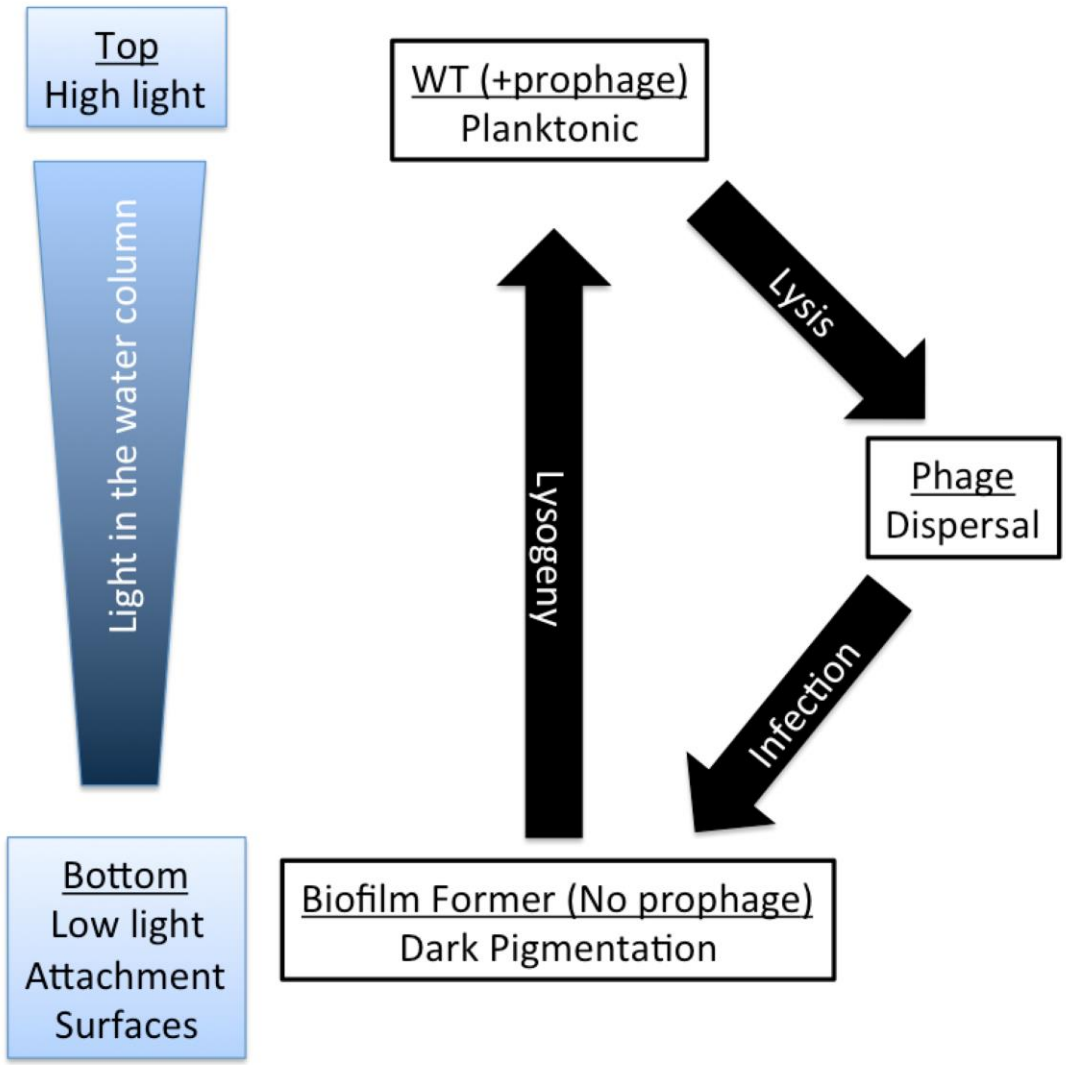

Figure 2- 3. A model of the prophage life cycle in *Synechococcus elongatus* PCC 7942 in natural environments.

2.5 Reference

1.Grigorieva, G.A. and S.V. Shestakov. *Application of the genetic transformation method for taxonomic analysis of unicellular blue-green algae*. Proc 2nd Int Symp Photosynthetic Prokaryotes, edited by G.A. Codd and W.P.D. Stewart. University of Dundee, Scotland. 1976

2.Golden, S.S. and L.A. Sherman. "Optimal conditions for genetic transformation of the cyanobacterium Anacystis nidulans R2." *Journal of Bacteriology* 158, no. 1 (1984): 36-42

3.Golden, S.S., J. Brusslan, and R. Haselkorn. "Expression of a family of psbA genes encoding a photosystem II polypeptide in the cyanobacterium Anacystis nidulans R2." *The EMBO journal* 5, no. 11 (1986): 2789-2798

4.Clerico, E.M., J.L. Ditty, and S.S. Golden. "Specialized techniques for site-directed mutagenesis in cyanobacteria." *Methods in molecular biology (Clifton, N.J.)* 362, no. 11 (2007): 155-171. doi: 10.1007/978-1-59745-257-1_11

5.Chen, Y., C.K. Holtman, A. Taton, and S.S. Golden. "Functional analysis of the synechococcus elongatus PCC 7942 Genome." In *Advances in Photosynthesis and Respiration*, edited by R.L. Burnap and W.F.J. Vermaas. p. 119-137. Springer Science+Business Media B.V. . 2012

6.Huang, H.H., D. Camsund, P. Lindblad, and T. Heidorn. "Design and characterization of molecular tools for a Synthetic Biology approach towards developing cyanobacterial biotechnology." *Nucleic Acids Research* 38, no. 8 (2010): 2577-2593. doi: 10.1093/nar/gkq164

7.Holtman, C.K., Y. Chen, P. Sandoval, A. Gonzales, M.S. Nalty, T.L. Thomas, P. Youderian, and S.S. Golden. "High-throughput functional analysis of the Synechococcus elongatus PCC 7942 genome." *DNA research : an international journal for rapid publication of reports on genes and genomes* 12, no. 2 (2005): 103-115. doi: 10.1093/dnares/12.2.103

8.Schatz, D., E. Nagar, E. Sendersky, R. Parnasa, S. Zilberman, S. Carmeli, Y. Mastai, E. Shimoni, E. Klein, O. Yeger, Z. Reich, and R. Schwarz. "Self-suppression of biofilm formation in the cyanobacterium

Synechococcus elongatus." *Environmental Microbiology* 15, no. 6 (2013): 1786-1794. doi: 10.1111/1462-2920.12070

9.Niederholtmeyer, H., B.T. Wolfstädter, D.F. Savage, P.A. Silver, and J.C. Way. "Engineering cyanobacteria to synthesize and export hydrophilic products." *Applied and Environmental Microbiology* 76, no. 11 (2010): 3462-3466. doi: 10.1128/AEM.00202-10

10.Huang, S., K. Wang, N. Jiao, and F. Chen. "Genome sequences of siphoviruses infecting marine Synechococcus unveil a diverse cyanophage group and extensive phage-host genetic exchanges." *Environmental Microbiology* 14, no. 2 (2012): 540-558. doi: 10.1111/j.1462-2920.2011.02667.x

11.Sugita, C., K. Ogata, M. Shikata, H. Jikuya, J. Takano, M. Furumichi, M. Kanehisa, T. Omata, M. Sugiura, and M. Sugita. "Complete nucleotide sequence of the freshwater unicellular cyanobacterium Synechococcus elongatus PCC 6301 chromosome: gene content and organization." *Photosynthesis research* 93, no. 1-3 (2007): 55-67. doi: 10.1007/s11120-006-9122-4

12.Yu, J., M. Liberton, P.F. Cliften, R.D. Head, J.M. Jacobs, R.D. Smith, D.W. Koppenaal, J.J. Brand, and H.B. Pakrasi. "Synechococcus elongatus UTEX 2973, a fast growing cyanobacterial chassis for biosynthesis using light and CO_2 ." *Scientific reports* 5, (2015): 8132. doi: 10.1038/srep08132

13.Watanabe, S., R. Ohbayashi, Y. Shiwa, A. Noda, Y. Kanesaki, T. Chibazakura, and H. Yoshikawa. "Light-dependent and asynchronous replication of cyanobacterial multi-copy chromosomes." *Molecular Microbiology* 83, no. 4 (2012): 856-865. doi: 10.1111/j.1365-2958.2012.07971.x

Chapter 3

Removal of the Prophage Enables Biofilm Formation

Because we observed that the lysogenic WT remains planktonic and the phage-less Δ50kb forms biofilms, we hypothesized that the prophage is repressing biofilm formation and that removal of the prophage or specific prophage genes will enable biofilm formation. Alternatively, SNPs present in the genome of Δ50kb that differ from the lab WT sequence may be responsible for this phenotype.

To test these hypotheses, I deleted the prophage from the Golden lab's WT strain, AMC06, in order to recreate the phage-less strain with a genetic background that lacks the Δ50kb-associated SNPs. This paper will describe the construction of the prophage deletion strain and show that removal of the prophage enables biofilm formation (Chapter 3). I also generated sigma factor mutants, related to one of the SNPs that was a candidate for causing the biofilm phenotype, and tested for biofilm formation (Chapter 4). Together, these data demonstrate that it is the prophage, not the sigma factor, that represses biofilm formation. To determine which genes in the prophage regulate this behavior, I will describe the deletion of sets of genes or individual genes in order to determine which prophage regions, genes, or non-coding RNAs actively repress biofilm formation (Chapter 5).

3.1 Generating the phage-less strain

The chromosomal difference between the Golden lab's WT strain, AMC06, and ∆50kb is diagrammatically demonstrated in **Figure 3- 1**, where two identical 60 bp attachment sites flank the prophage in WT while only a single attachment site is present in the ∆50kb strain. In theory, induction and/or excision could yield the desired prophage-less strain from a WT genetic background. However, attempts by Dr. Simkovsky to use canonical methods of induction, including UV irradiation, mitomycin C, and metal toxicity did not appear to induce the prophage and the natural mechanism of induction is still currently unknown. Although Dr. Simkovsky has since been able to use genetic mechanisms for inducing excision of the prophage genome and lysis of the cells, those tools were not available at the time to enable deletion of the prophage.

Because markerless deletion of the prophage would require a difficult series of "hit-and-run" transformations and counter-selections to ensure complete removal of all chromosomes containing the prophage, we decided to approximate the difference between WT and ∆50kb by replacing the prophage region with an antibiotic-resistance cassette flanked by the two 60 bp attachment sites. This same final construct was introduced into both WT and ∆50kb to generate strains, referred to as WTdel and ∆50del respectively, as appropriate for phenotypic comparison.

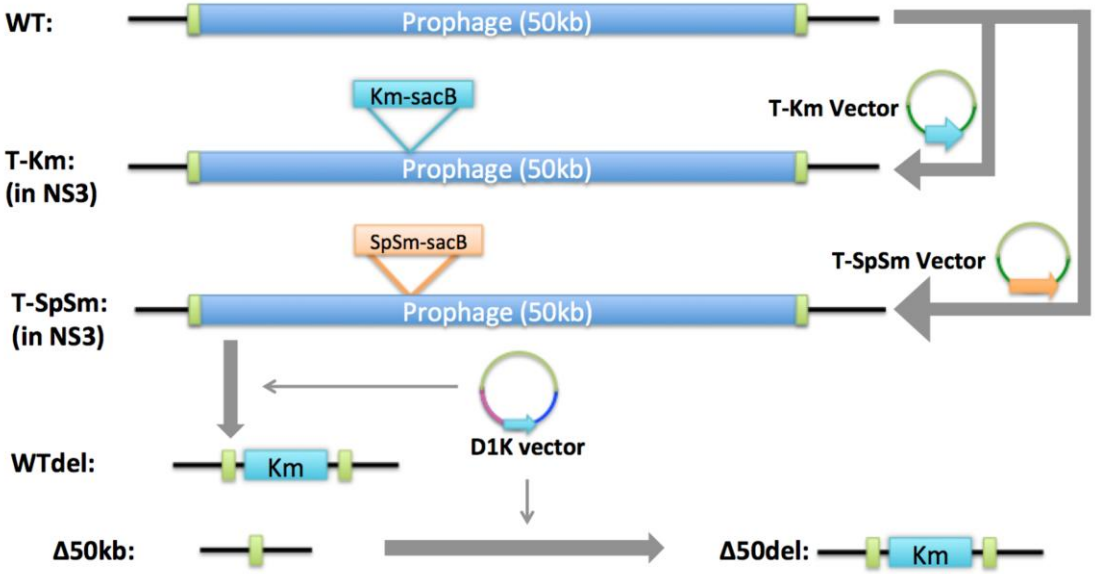

Figure 3- 1. Multistep approach of generating the phage-less strain. In the first step, either a kanamycin (Km-) or a spectinomycin and streptomycin (SpSm-) resistance gene was inserted along with a *sacB* expression cassette into WT at NS3, which is located in the prophage. Subsequently, the SpSm-tagged prophage was replaced with a Km-resistant cassette, generating the phage-less strain WTdel. Δ50kb was transformed with the same deletion vector, D1K, from the previous step to generate Δ50del. The prophage insertion region of WTdel and Δ50del are identical.

The final deletion vector, D1K, encoding a kanamycin cassette, was generated via seamless assembly of standardized parts developed by Dr. Arnaud Taton in the Golden lab [1] and PCR amplicons containing the prophage flanking regions (**Figure 3- 2**). To generate assembly-acceptable amplicons that serve as homologous recombination regions, I performed PCR using primers that would enable these regions to be assembled with a Km-resistance part derived from pCVD012 and an *E. coli* origin of replication part derived from pCVD028 from the Golden lab assembly system using the Life Technologies Seamless Assembly Kit [1]. Assembled vectors were transformed into *E. coli* and verified by PCR, restriction endonuclease digestion analysis, and sequencing. All other deletion vectors discussed in this thesis were generated using the same methodology with only the primers and target amplicons being changed to generate the desired deletion vector. Another vector, D1C, was similarly created through assembly to delete the prophage without leaving an antibiotic-resistance cassette, thus resulting in the exact genome structure found in ∆50kb.

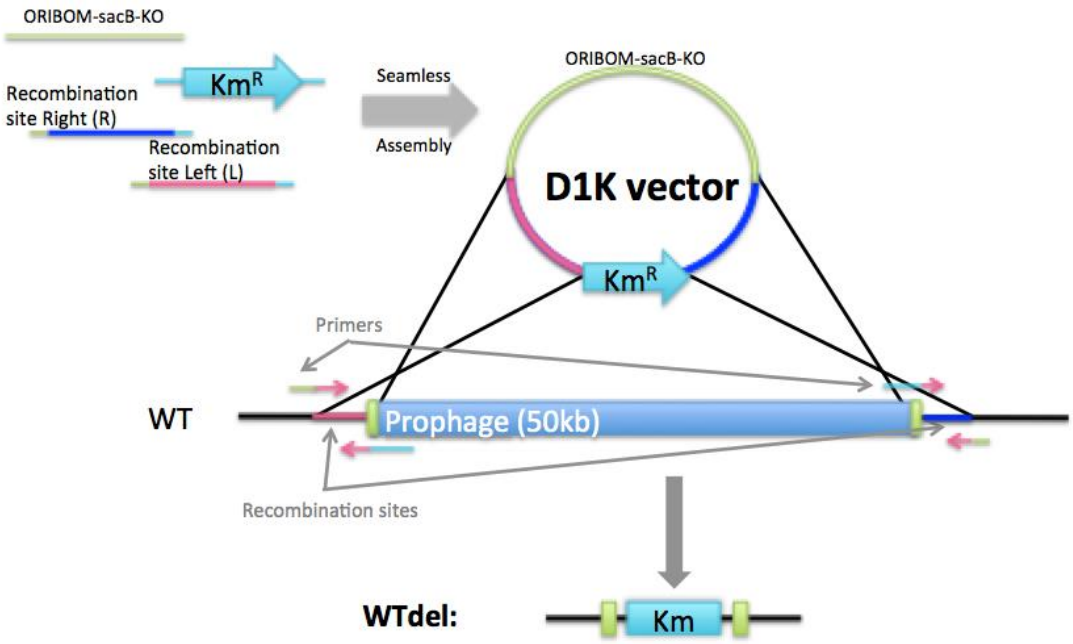

Figure 3- 2. Deletion vector D1K design. The regions surrounding the prophage known as recombination sites were PCR amplified by using primers that allow for assembly with a kanamycin-resistance part and an *E. coli* origin of replication part derived via digestion from the Golden lab's seamless assembly system. Through double homologous recombination, this vector can replace the prophage with a kanamycin-resistance cassette. Following transformation and recombination, the two attachment sites remain surrounding the antibiotic resistance cassette.

Successfully constructed deletion vectors were transformed into WT *S. elongatus* PCC 7942, AMC06, in order to generate the deletion strain [2]. Unfortunately, all attempts at creating a phage-less strain from AMC06 in a single step using either D1K or D1C failed to generate a reliable, segregated clone that completely lacked the prophage from all copies of the chromosome. Thus, it was necessary to take a multistep approach in which we took advantage of the previously designed neutral site 3 to tag the prophage with a counter-screenable antibiotic-resistance cassette and *sacB*, a counter-selectable marker that makes cells lethally sensitive to sucrose. Neutral site 3 is located in SynPCC7942_0739 in the tail section (section 5) of the prophage [3]. This gene putatively encodes the gp25-like baseplate wedge and lysozyme protein and does not appear to be expressed in the WT lysogen [4].

Seamless assembly was again used to generate the vectors necessary to tag the prophage, though these tagging vectors were created through a two-step protocol (**Figure 3- 3**). First, a pre-tagging vector was constructed from 3 parts directly from the Golden lab's seamless assembly: a neutral site 3 recombination part with an *E. coli* origin of replication derived from pCVD024, an antibiotic resistance part derived from either pCVD002 (spectinomycin and streptomycin-resistance, SpSm) or pCVD012 (kanamycin-resistance, Km), and a cloning cassette containing the suicide gene *ccdB* derived from pCVD015. Two pre-tagging vectors were thus generated, NS3-ccdB-swaI-Km and NS3-ccdB-swaI-SpSm, and transformed into DB3.1 *E. coli* cells that can

tolerate the presence of CcdB. In a second assembly step, the *ccdB* casettes were replaced via digestion of the pre-tagging vectors with swaI and assembly with a PCR amplicon containing a SacB expression cassette derived from pCVD0028 with appropriate 25nt-adaptor sequences to enable assembly. Thus, two tagging vectors, T-Km and T-SpSm, were generated and confirmed via restriction digest, PCR, and sequencing.

These tagging vectors were transformed into AMC06 to generate the T-Km and T-SpSm *S. elongatus* strains. Double homologous recombination and complete segregation of the tagged chromosomes were confirmed via PCR. Segregation and the functionality of the *sacB* gene product were further confirmed via a failure of the T-Km and T-SpSm strains to grow on plates containing 5% sucrose (**Figure 3- 4**).

Subsequently, the SpSm-tagged PCC 7942 strain T-SpSm was transformed with the D1K vector in order to replace the entire prophage with a Km-resistant cassette. Transformants were selected on plates containing BG11+Km+5% sucrose to counter-select against the tagged prophage chromosomes. Counter-screening against the SpSm-resistant cassette was also performed (**Figure 3- 5**). This counter-screen proved an invaluable and reliable method to confirm segregation, as many clones that survived on sucrose also survived on SpSm, indicating both the presence of the tagged prophage chromosome and a possible suppressor mutation of the *sacB* gene. Only those clones that died on SpSm and survived in the presence of Km and sucrose were further checked via PCR to confirm the complete absence of the

prophage. The successfully generated phage-less strain is here after referred to as WTdel. The same D1K vector from this final step was used in order to generate a Km-tagged ∆50kb strain, referred to as ∆50del, which is identical to WTdel in the prophage region. Since Km-tagged WT, WTdel, and ∆50del are all Km-resistant, all three were used to control for the effects of antibiotics in subsequent phenotyping experiments.

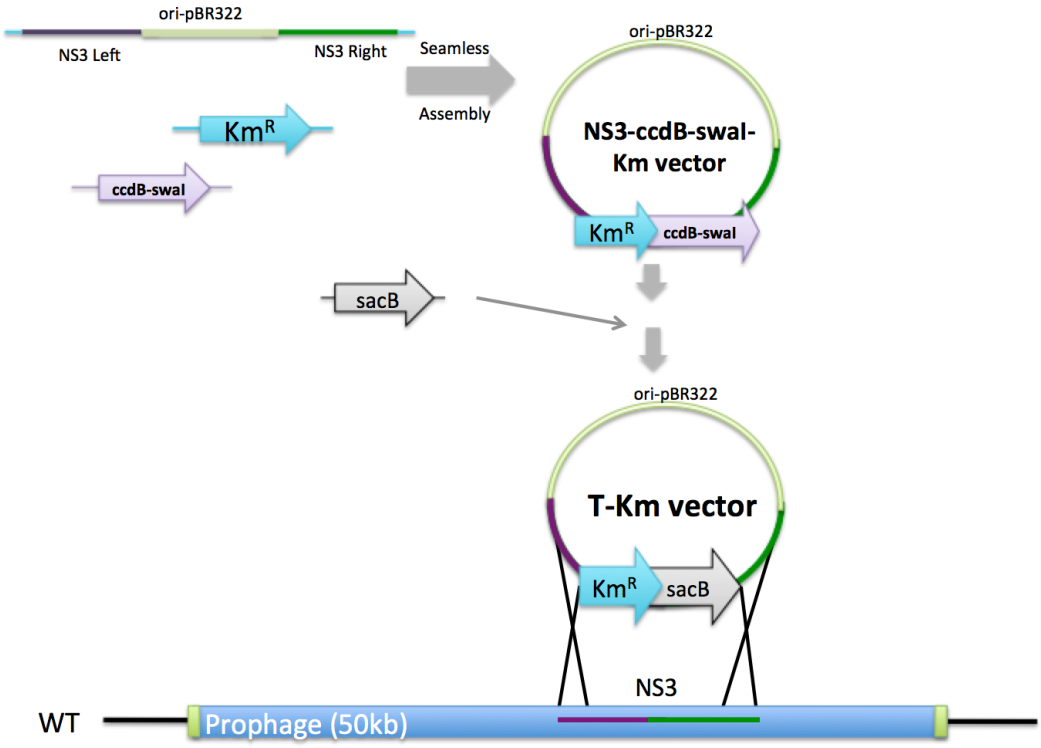

Figure 3- 3. Tagging vector T-Km design. The pre-tagging vector was designed by connecting a Km-resistant part, a ccdB-swaI part and an *E.coli* origin of replication with the regions surrounding NS3. The ccdB-swaI was then replaced with sacB part as counter selection.

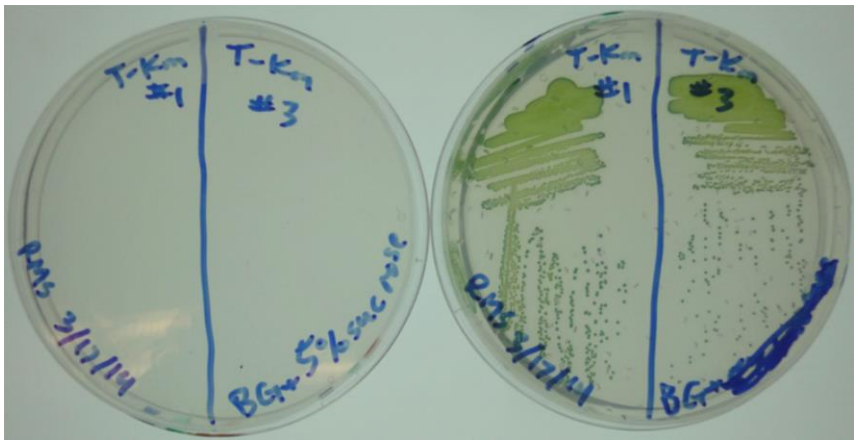

Figure 3- 4. Sucrose sensitivity test of the tagged prophage strain T-Km. A kanamycin resistance cassette and *sacB* expression cassette were inserted into neutral site 3 of WT to generate T-Km. Two clones of T-Km were grown on solid BG11 media supplemented with (left) or without (right) 5% sucrose. The inability of these clones to grow on sucrose indicates the integration and function of the *sacB* expression cassette.

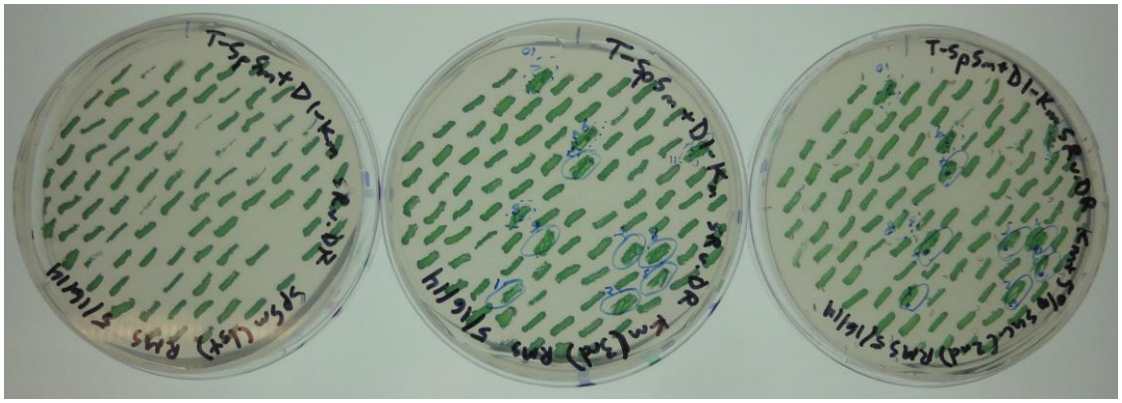

Figure 3- 5. Antibiotic resistance counter-screening of WTdel clones. WTdel clones were generated through transformation of the prophage-deletion vector D1K into the T-SpSm strain. Individual clones were replica plated as streaks onto solid BG11 media supplemented with (left) spectinomycin and streptomycin (SpSm), (center) kanamycin (Km), or (right) kanamycin and 5% sucrose. Clones that grew on all three plates retain chromosomes with tagged prophage and chromosomes with the prophage replaced by a kanamycin cassette. Circled clones on the center and right plates are clones that are sensitive to SpSm and therefore represent the desired WTdel mutant that lacks any of the parental T-SpSm chromosomes.

3.2 The prophage represses biofilm formation

The confirmed phage-less strain WTdel forms a biofilm that attaches to the bottom of glass flasks and tubes, while WT does not attach to the flask surface (**Figure 3- 6A**). Upon quantifying the biofilm formation from numerous biological replicates using the crystal violet staining and absorbance measurement protocol of Fisher, et al. [5], it is clear that WTdel forms biofilms to a similar extent as the published biofilm-forming mutant, GspEΩ [6] (**Figure 3-6B**). Consistent with our previous observations, ∆50kb and ∆50del also formed films. In contrast, T-Km and T-SpSm strains did not form visible biofilms and produced similar crystal violet absorbance readings as WT. These data rule out the possibility that biofilm formation is dependent upon the application of a particular antibiotic or the expression of the antibiotic cassette. Instead, these data support the conclusion that the prophage, rather than any of the SNPs present in ∆50kb, is quantitatively responsible for repressing biofilm formation. Moreover, the prophage gene into which the antibiotic-resistance cassettes are inserted in T-Km and T-SpSm have no effect on the biofilm phenotype.

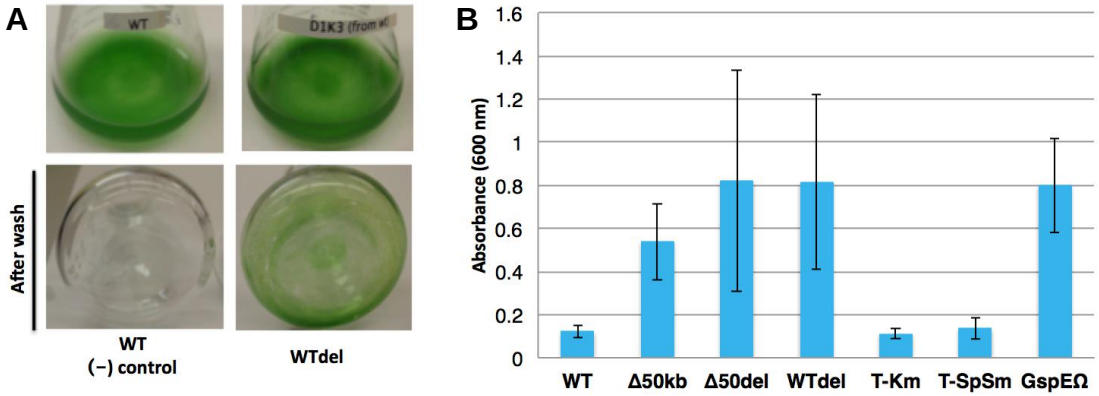

Figure 3- 6. The deletion of the prophage allows biofilm formation. (A)The phage-less strain WTdel forms a biofilm that remains attached to the flask after three water washes. (B) Flask cultures were allowed to form biofilms, which were quantified by crystal violet staining measured via absorbance at 600 nm of stain extracted from the films. Strains analyzed include WT as a negative control, the tagged prophage strains T-Km and T-SpSm, GspEΩ as a positive control for biofilm formation, and the phage-less strains Δ50kb, Δ50del, and WTdel.

3.3 A new isolate of *S. elongatus,* WC-1, validates *S. elongatus*' natural ability to form films and supports the role of the prophage in regulating planktonicity

In collaboration with Dr. Jerry Brand's group at the University of Texas in Austin, we recently received a newly isolated strain of *S. elongatus*, called WC-1. Dr. Brand's group isolated this strain from Waller Creek, where some of the original strains of *S. elongatus* were isolated. While the genome of this strain has not yet been sequenced, we have confirmed that WC-1 completely lacks the prophage and has a genomic structure at this locus that resembles ∆50kb. As seen in **Figure 3- 7C**, WC-1 readily forms more extensive biofilms than any other tested strain. Also, the biofilm of WC-1 clearly formed in a light-dependent directional manner, with increased biofilm formation in the darker regions of the flask than in the part of the flask closer to the light (**Figure 3- 7C**). This finding indicates that this strain may also be phototactic, whereas our laboratory WT strain is not. These data support the model that *S. elongatus* is naturally capable of forming biofilms, that the prophage represses biofilm formation, and that the laboratory strain likely underwent selective pressure through domestication to not form biofilms and to retain the prophage.

Chapter 3, in full, is currently being prepared for submission for publication of the material. Simkovsky, Ryan; Golden, Susan. The thesis author was the primary investigator and author of this material.

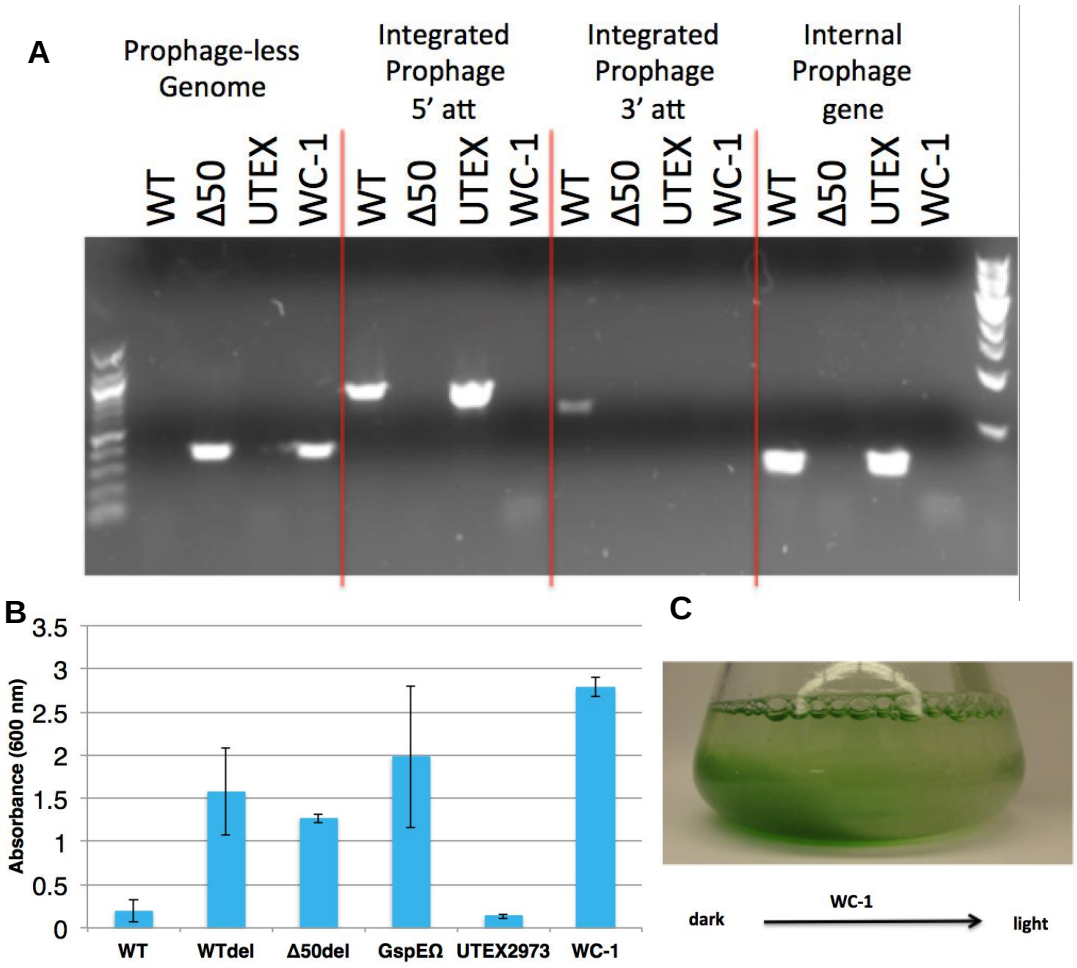

Figure 3- 7. The newly isolated *S. elongatus* strain WC-1 from Waller Creek in Austin, Texas lacks the prophage and forms strong biofilms.(A) WC-1 was confirmed as lack of the prophage by PCR performed by Dr. Simkovsky. (B) Flask cultures were allowed to form biofilms, which were quantified by crystal violet staining measured via absorbance at 600 nm of stain extracted from the films. WTdel, Δ50del and WC-1 do not encode the prophage in their genomes, while WT encodes the entire prophage and UTEX 2973, a fast growing strain of *S. elongatus* [7], encodes nearly the entire prophage. For data point, n≥3 (C) WC-1 biofilms appeared to form in a gradient, with more dense films forming away from the light source.

3.4 Reference

1.Taton, A., F. Unglaub, N.E. Wright, W.Y. Zeng, J. Paz-Yepes, B. Brahamsha, B. Palenik, T.C. Peterson, F. Haerizadeh, S.S. Golden, and J.W. Golden. 2014. "Broad-host-range vector system for synthetic biology and biotechnology in cyanobacteria." *Nucleic Acids Research*. Vol. 42(17): e136-e136. doi:10.1093/nar/gku673

2.Golden, S.S., J. Brusslan, and R. Haselkorn. 1987. "Genetic engineering of the cyanobacterial chromosome." *Methods in enzymology*. Vol. 153: 215-231.

3.Niederholtmeyer, H., B.T. Wolfstädter, D.F. Savage, P.A. Silver, and J.C. Way. 2010. "Engineering cyanobacteria to synthesize and export hydrophilic products." *Applied and Environmental Microbiology*. Vol. 76(11): 3462-3466. doi:10.1128/AEM.00202-10

4.Vijayan, V., I.H. Jain, and E.K. O'Shea. 2011. "A high resolution map of a cyanobacterial transcriptome." *Genome biology*. Vol. 12(5): R47. doi:10.1186/gb-2011-12-5-r47

5.Fisher, M.L., R. Allen, Y. Luo, and R. Curtiss. 2013. "Export of extracellular polysaccharides modulates adherence of the Cyanobacterium synechocystis." *PloS one*. Vol. 8(9): e74514. doi:10.1371/journal.pone.0074514

6.Schatz, D., E. Nagar, E. Sendersky, R. Parnasa, S. Zilberman, S. Carmeli, Y. Mastai, E. Shimoni, E. Klein, O. Yeger, Z. Reich, and R. Schwarz. 2013. "Self-suppression of biofilm formation in the cyanobacterium Synechococcus elongatus." *Environmental Microbiology*. Vol. 15(6): 1786-1794. doi:10.1111/1462-2920.12070

7.Yu, J., M. Liberton, P.F. Cliften, R.D. Head, J.M. Jacobs, R.D. Smith, D.W. Koppenaal, J.J. Brand, and H.B. Pakrasi. 2015. "Synechococcus elongatus UTEX 2973, a fast growing cyanobacterial chassis for biosynthesis using light and CO_2 ." *Scientific reports*. Vol. 5: 8132. doi:10.1038/srep08132

Chapter 4

Phenotypic Effects of A Sigma Factor Mutation

4.1 Generating a sigma factor mutant.

As stated in Chapter 2, reanalysis of the published sequencing data for the Δ50kb strain revealed that the most obviously significant SNP outside of the prophage region is one that results in the truncation of the sigma factor gene *sigF* (Synpcc7942_1510). In *Synechocystis sp.* strain PCC 6803, SigF enables phototaxis and motility through the expression of the type IV pilus-forming *pilA* genes [1] and plays a role in acclimation to salt or high light stress [2], likely through alterations in cell surface properties. Because our collaborators in the Schwarz lab have evidence that knocking out *pilA* genes can induce biofilm formation in PCC 7942, we hypothesized that a truncation of this gene could be responsible for or contribute to the ∆50kb biofilm phenotype. Because this truncation is in the middle of the second DNA-binding domain of Synpcc7942_1510 (**Figure 4- 1**), it was unclear if this mutation results in a loss or a gain of function through the loss of binding-specificity domains. To determine the impact of ∆50kb's *sigF* truncation, mutants were generated in the backgrounds of WT, WTdel, and ∆50kb via transformation of two different transposon insertional vectors from the Golden lab's Unigene Set (UGS) [3, 4] private mutant library: 4E8-N5, referred to as N5, in which the insertion is located in the N-terminal-coding end of the gene to generate a loss-of-function

knockout, and 1E9-D7, referred to as D7, in which the insertion is in the third DNA-binding domain (**Figure 4- 1**). Segregated mutant clones were confirmed by PCR analysis.

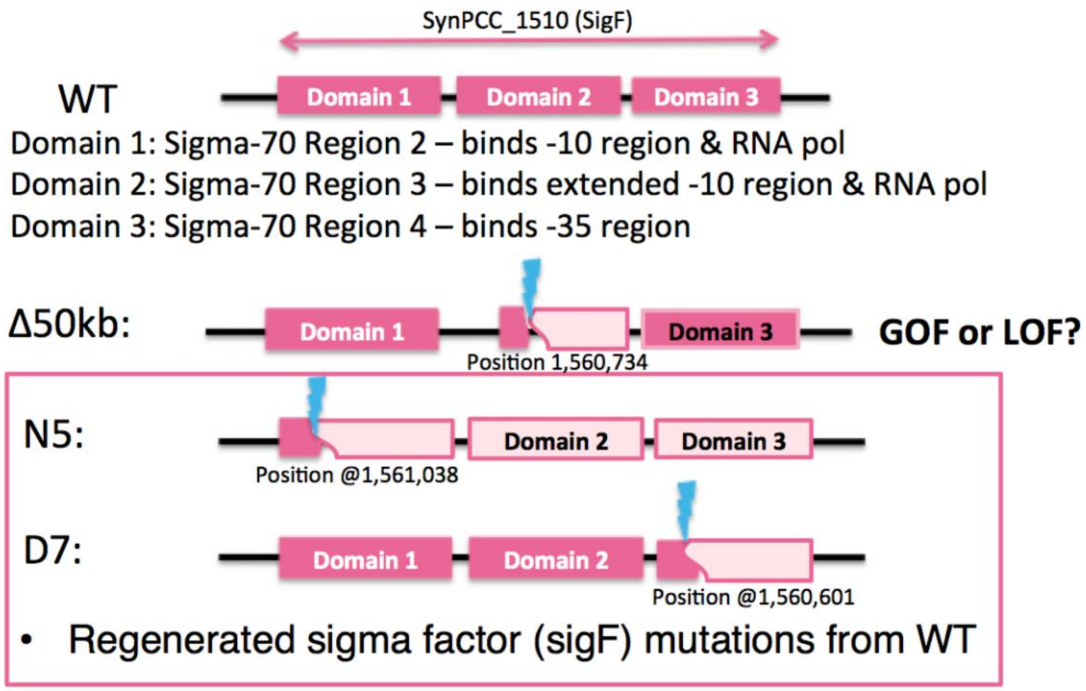

Figure 4- 1. Mutations in sigma factor sigF. In Δ50kb, the SigF protein is truncated in the middle of domain 2 of Synpcc7942_1510. The mutants generated from the UGS library from WT PCC 7942 are either a knockout the entire gene known as N5 or the final domain D7.

4.2 *sigF* mutants settle, but do NOT enhance biofilm formation

After growth but prior to the initiation of the biofilm formation assay (see appendix for assay details), the parental strains and their *sigF* mutants were observed for the ability to settle out of suspension, as opposed to remaining planktonic. As expected, WT remained planktonic while ∆50kb settled (**Figure 4-2**). Interestingly, WTdel remained planktonic during this time period. In contrast, all *sigF* mutants settled to the bottom of the culture. These data indicate that *sigF* enables the planktonic state of the strain while the prophage does not impact this phenotype.

Quantification of biofilm formation demonstrated that the *sigF* mutations do not enable biofilm formation (**Figure 4- 3**). Neither of the *sigF* mutations in the genetic background of WT significantly increases biofilm formation, as determined by crystal violet staining and absorbance measurements, over that of WT. In contrast to the hypothesis that *sigF* mutations enable biofilm formation, both *sigF* mutants in the WTdel background appear to form decreased amounts of biofilm compared to WTdel, though these values are still significantly higher than that observed for both WT and the published biofilm former, GspEΩ. In ∆50kb, these mutations do enhance biofilm formation but not more than in ∆50del, indicating a degree of biofilm variability in the ∆50kb strains that may be associated with the presence of an antibiotic-resistance gene as opposed to the *sigF* mutations. Overall, these data suggest that the prophage represses biofilm formation, while the sigma factor activates or maintains the planktonic state.

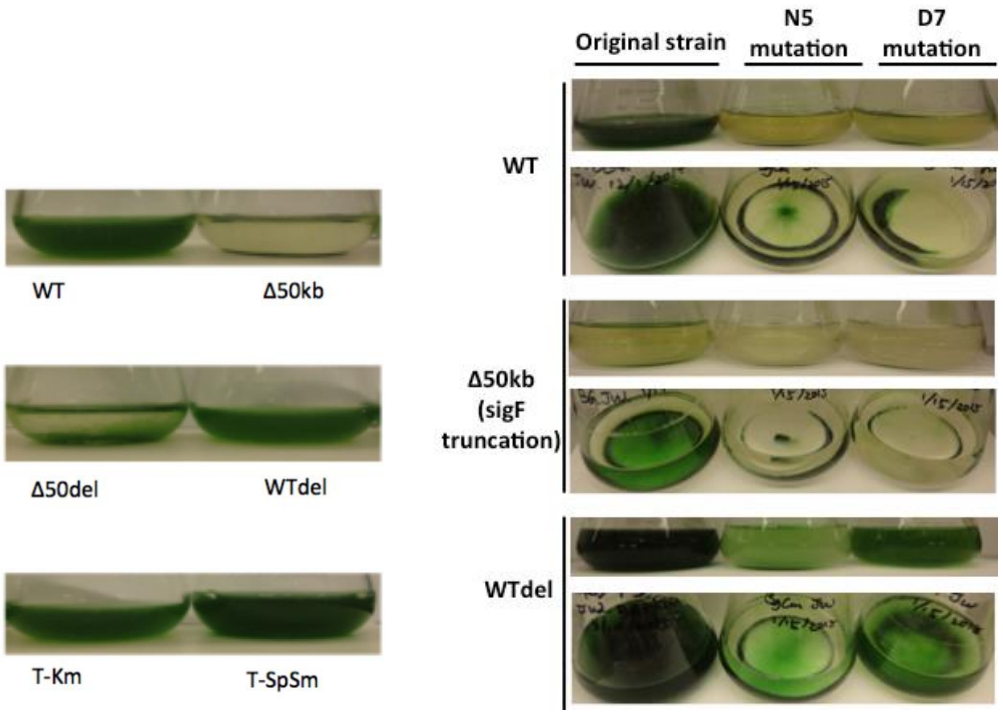

Figure 4- 2. SigF mutations cause settling in flasks stored on the bench. The parental strains are WT, ∆50kb (which encodes a SigF truncation) and phage-less WTdel. N5 mutants possess an insertional knockout of domain 1 of *sigF* while D7 mutants have a transposon insertion in domain 3 of *sigF*.

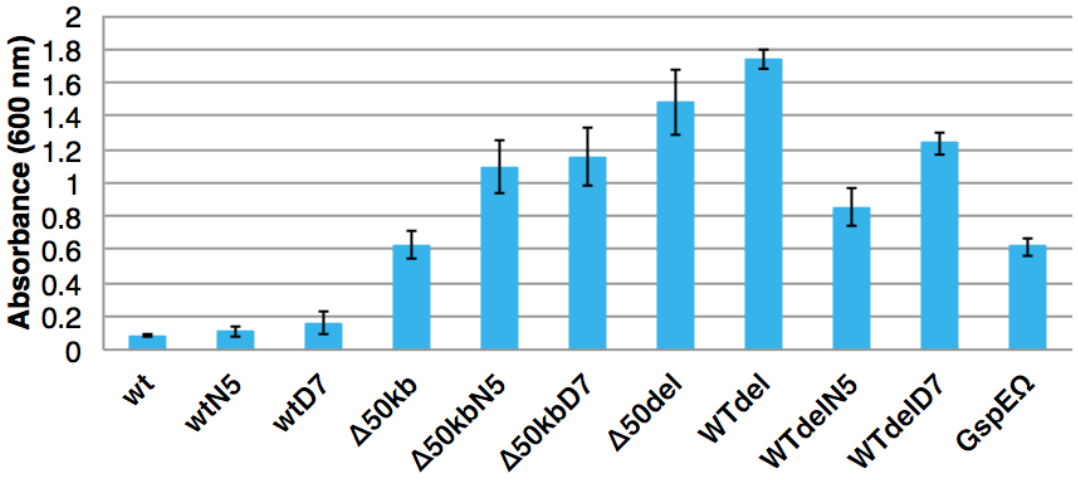

Figure 4- 3. Biofilm analysis of *sigF* mutants. Flask cultures were allowed to form biofilms, which were quantified by crystal violet staining measured via absorbance at 600 nm of stain extracted from the films. The parental strains are WT, ∆50kb (which encodes a SigF truncation) and phage-less WTdel. N5 mutants possess an insertional knockout of domain 1 of *sigF* while D7 mutants have a transposon insertion in domain 3 of *sigF*. GspEΩ was assayed as a positive control. For each data point, n≥3.

4.3 *sigF* mutations rescue the light:dark growth defect of RpaA-deficient cells

It is known that the deletion of the *rpaA* gene (ΔrpaA), which encodes a component of the output mechanism of the circadian clock in PCC 7942, results in mutant strains that are not capable of forming colonies when grown in light:dark cycles [5], a phenotype referred to as LD-sensitivity. Preliminary evidence had suggested that this LD-sensitivity is the result of active cell death in response to changes in light, rather than impaired growth [6]. Dr. Simkovsky tested the hypothesis that the prophage or its induction is the active agent of cell death in ΔrpaA mutants by deleting the *rpaA* gene from the Δ50kb and WTdel strains and testing these mutants for survival in the dark. While Δ50kb-ΔrpaA^{-} mutants survived and grew in LD cycles, ΔrpaA mutants of WTdel did not. These results indicate that one of the Δ50kb SNPs suppresses the LD-sensitivity phenotype.

To test the hypothesis that mutations in *sigF* rescue the LD-sensitivity phenotype of ΔrpaA, I generated ΔrpaA mutants in the backgrounds of WT and WT's *sigF* mutants. Mutants and parental strains were then assayed for the LD-sensitivity phenotype using serial dilution spot plates to check for culture and colony growth on plates under light:dark cycles (**Figure 4- 4**). As expected in this assay, WT+ΔrpaA is impaired in growth under a 12:12 LD cycle but is not growth impaired when grown under constant light. In contrast, the introduction of either *sigF* mutation rescues the WT+ΔrpaA LD-sensitivity phenotype and enables colony growth similar to that of WT. These data support the conclusion

that the *sigF* truncation is responsible for the suppression of LD-sensitivity in ∆50kb+∆rpaA.

4.4 Summary

In this chapter, I have demonstrated that mutation of the *sigF* gene in *S. elongatus* causes settling of the cells to the bottom of the flask, does not enhance the ability of the cell to form biofilms, and is responsible for rescue of the ∆rpaA LD-sensitivity phenotype. These data further support the hypothesis that the biofilm formation phenotype observed in ∆50kb and WTdel is due to the lack of the prophage rather than one of the SNPs identified in ∆50kb. Although the *sigF* truncation in ∆50kb causes cells to settle, likely due to a resulting lack of pili as observed in *Synechocysistis* sp. PCC 6803, it does not appear to determine biofilm formation. Instead, the *sigF* mutation may impact the kinetics of film formation via the rapidity of accumulation of cells at the bottom of the flask.

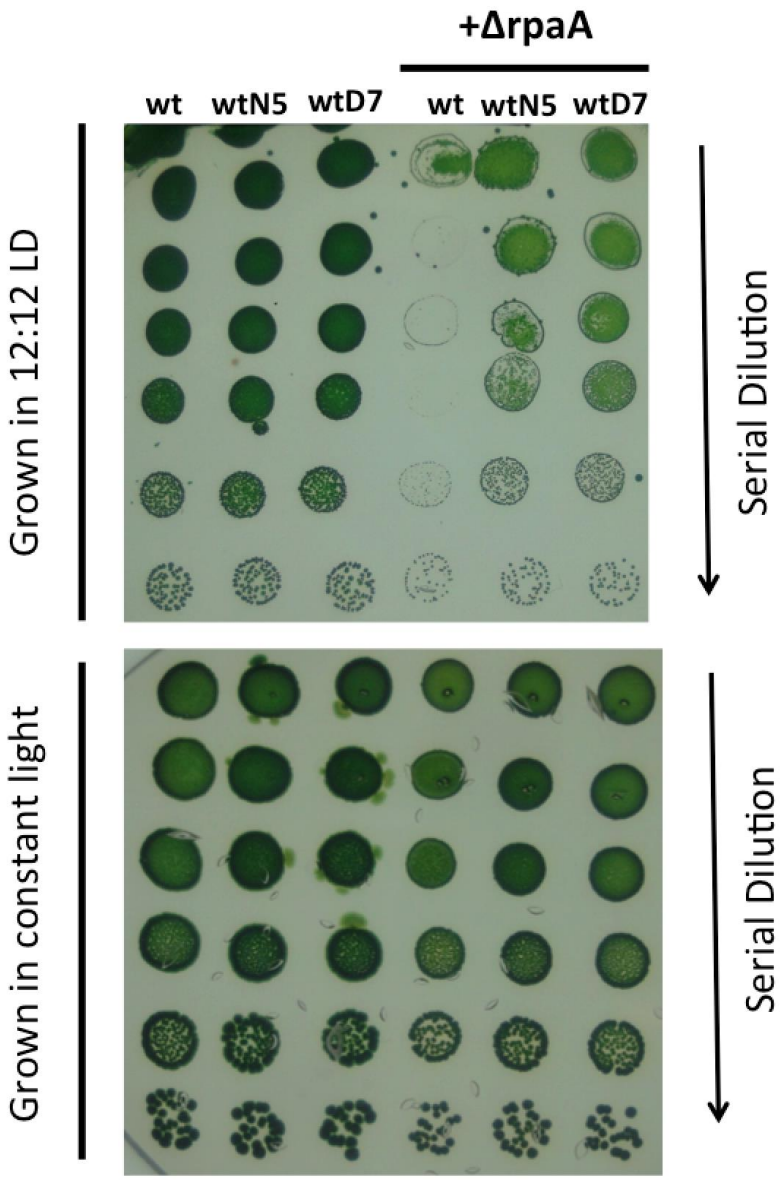

Figure 4- 4. Light:dark growth sensitivity assay of ΔrpaA mutants. Cultures were serially diluted at 1:5 dilution (original culture at top, most dilute culture at bottom for each plate) and a 5 µl drop of each dilution was spotted onto solid BG11 media. Cultures were grown in a 12:12 light:dark cycle (top) or under constant light (bottom). All cultures grew under constant light, while WT+ΔrpaA did not grow in LD. WTN5 = WT + N5 *sigF* mutation; WTD7 = WT + D7 *sigF* mutation.

4.5 Reference

1.Bhaya, D., N. Watanabe, T. Ogawa, and A.R. Grossman. "The role of an alternative sigma factor in motility and pilus formation in the cyanobacterium Synechocystis sp. strain PCC6803." *Proc Natl Acad Sci U S A* 96, no. 6 (1999): 3188-3193

2.Huckauf, J., C. Nomura, K. Forchhammer, and M. Hagemann. "Stress responses of Synechocystis sp. strain PCC 6803 mutants impaired in genes encoding putative alternative sigma factors." *Microbiology (Reading, England)* 146, no. 11 (2000): 2877-2889. doi: 10.1099/00221287-146-11-2877

3.Chen, Y., C.K. Holtman, A. Taton, and S.S. Golden. "Functional analysis of the synechococcus elongatus PCC 7942 Genome." In *Advances in Photosynthesis and Respiration*, edited by R.L. Burnap and W.F.J. Vermaas. p. 119-137. Springer Science+Business Media B.V. . 2012

4.Holtman, C.K., Y. Chen, P. Sandoval, A. Gonzales, M.S. Nalty, T.L. Thomas, P. Youderian, and S.S. Golden. "High-throughput functional analysis of the Synechococcus elongatus PCC 7942 genome." *DNA research : an international journal for rapid publication of reports on genes and genomes* 12, no. 2 (2005): 103-115. doi: 10.1093/dnares/12.2.103

5.Takai, N., M. Nakajima, T. Oyama, R. Kito, C. Sugita, M. Sugita, T. Kondo, and H. Iwasaki. "A KaiC-associating SasA-RpaA two-component regulatory system as a major circadian timing mediator in cyanobacteria." *Proceedings of the National Academy of Sciences of the United States of America* 103, no. 32 (2006): 12109-12114. doi: 10.1073/pnas.0602955103

6.Diamond, S. "Circadian and Diurnal Control of Metabolism in Cyanobacteria." PhD diss., University of California, San Diego, 2015

Chapter 5

Investigating Which Prophage Gene(s) Repress Biofilm Formation

Our evidence so far supports a role for the prophage in biofilm formation. Genetic and phenotypic comparison of ∆50kb, WTdel, and WT indicates that the prophage does not passively repress biofilm formation through chromosomal disruption via integration, as the integration does not appear to disrupt any genetic elements and the presence of an antibiotic cassette at this locus does not repress biofilm formation. These observations support the hypothesis that the prophage expresses genes in the lysogenic state to actively repress biofilm formation. We wish to determine which genes in the prophage regulate this behavior.

5.1 Deletions of Gene Sections and Biofilm Analysis

Since there are over 50 genes in this prophage, we first proceeded to delete sections of genes encoding proteins of similar function, such as the region encoding the tail genes (section 5), in order to narrow down which genes or possibly non-coding RNAs repress biofilm formation. All section deletion vectors were constructed using a seamless assembly strategy similar to that for generating D1K (see Chapter 3). As an example, the assembly of the tail genes section 5 deletion vector, Ds5, was accomplished by using PCR to

amplify ~500 bp long recombination regions that abutted the start codon of the first gene and the stop codon of the last gene in the section 5 region. The primers used to amplify these recombination regions were designed to enable their assembly with a Km-resistance part derived from pCVD012 and an *E. coli* origin of replication part derived from pCVD028 (**Figure 5- 1**). Once the deletion vector was constructed, transformed into *E. coli*, and verified by PCR, restriction endonuclease analysis, and sequencing, the vector was used to transform and recombine with WT in order to generate the desired deletion strain, Ds5.

All *S. elongatus* prophage section deletion strains were verified by PCR to check for double recombination and segregation. For small deletions, a single pair of PCR primers is sufficient to detect the difference in size at the loci of interest between WT and the deletion mutant. However, for larger deletions such as Ds5, two PCR reactions using a total of three primers were required to properly verify the clone. In the first PCR reaction, primers binding to the recombination regions flanking the gene section detect only mutant chromosomes because the WT region is too large to produce a band. In the case of Ds5, correct mutants are expected to produce an amplicon 2,062 bp in size (**Figure 5- 2**). The second reaction is designed to detect WT chromosomes since it uses one of the primers that binds to the recombination region and a second primer that binds just inside of the deleted section. Thus, the combination of results from these two PCRs indicates if the mutation has been integrated into all copies of the genome.

I attempted to generate five section deletions and was successful in generating three of these mutants: Ds4, which deletes the section encoding the phage portal and head; Ds5, which deletes the phage tail section; and Ds7, which deletes six genes including those that encode the phage lysozyme, putative DNA binding proteins, and a predicted restriction endonuclease. The deletions of section 2 or section 3 were not successfully generated. Although transformation with the Ds2 vector produced hundreds of colonies, PCR analysis of these colonies demonstrated that they retained the WT chromosome. Transformation with Ds3 never produced any colonies. We propose that the failure to generate Ds2 or Ds3 is due to the presence of prophage gene, Synpcc7942_0723, shown to be essential for viability [1], at the transcriptional start of section 2 that may be necessary for maintaining the lysogenic state. This gene appears to be significantly expressed in WT [2, 3] and we hypothesize that its removal via Ds2 or the removal of its promoter via Ds3 results in either cell death or the excision of the phage genome in a way that destabilizes the chromosome. Because sections 1 and 6 encompass only a single gene each, the knockouts of these sections were investigated using UGS transposon insertional mutant vectors. Similarly, because sections 8 and 9 are predicted to impact the state switch between lysogeny and lysis, these genes were investigated using individual UGS mutants rather than deletion of the entire region. Individual UGS gene mutants will be discussed further in the next section.

Segregated clones of the section deletions Ds4, Ds5, and Ds7 were

analyzed using the biofilm assay protocol described in the appendix (**Figure 5-3**). All section deletions produced biofilms in quantities significantly different than WT. However, the amount of biofilm production in these section deletions is significantly less than when the entire prophage is deleted in WTdel. Due to the initial low values of biofilm production in ∆50kb and GspEΩ, the experiment was performed again. This experimental replicate confirmed the initial observation that the section deletions produce biofilms in greater quantities than WT but not to the same degree as the phage-less or GspEΩ biofilm-forming strains.

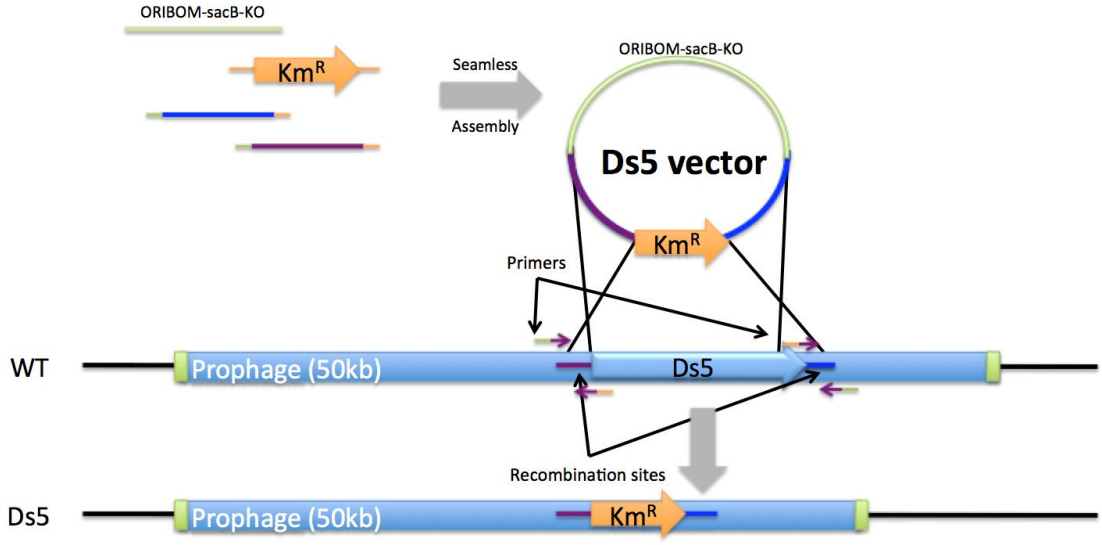

Figure 5- 1. Deletion vector design for removing all genes in section 5 encoding the phage tail. Recombination regions (~500 bp in length) surrounding section 5 were PCR amplified by using primers that allow them to be assembled with a Km-resistance part and an *E. coli* origin of replication part from Golden lab's seamless assembly system in order to generate the deletion vector. Transformation of this vector into WT allows for the replacement of all of the tail genes with a kanamycin resistance cassette.

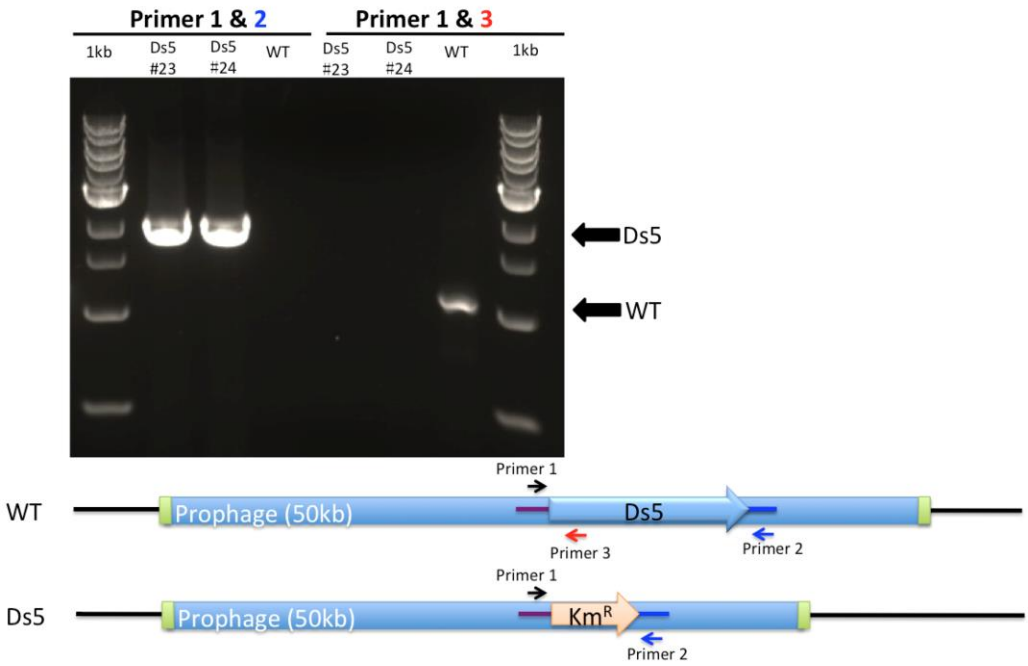

Figure 5- 2. The *Synechococcus* deletion strains of section 5 (Ds5) were verified by PCR. Primer 1 and Primer 2 were designed outside of section 5, while Primer 3 was designed inside of the section 5. Amplification of WT chromosomes with primers 1 and 2 should not produce a band due to the large size of section 5, while PCR on a chromosome lacking section 5 should produce a 2,062 bp band. In contrast, PCR with primers 1 and 3 will only produce a band from WT chromosomes. Gel electrophoresis analysis on a 0.7% agarose gel of WT and two Ds5 clones is shown above.

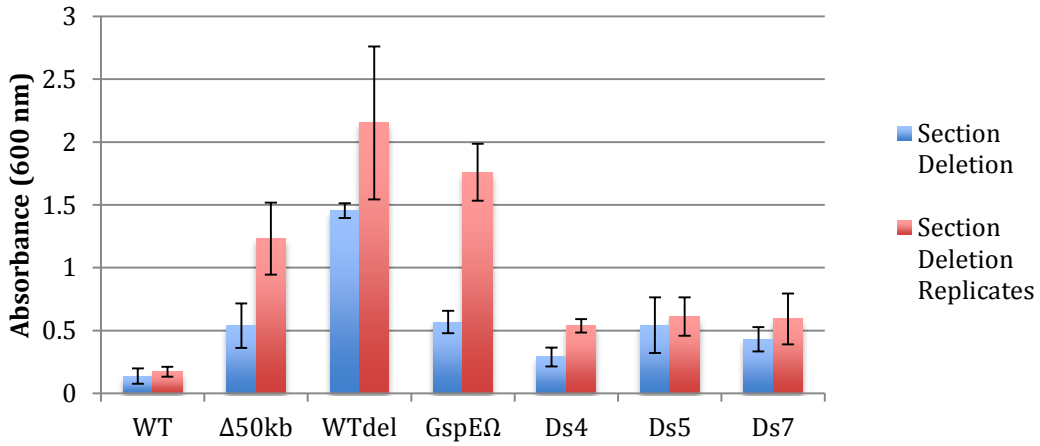

Figure 5- 3. Biofilm analysis of gene section deletions. Flask cultures were allowed to form biofilms, which were quantified by crystal violet staining measured via absorbance at 600 nm of stain extracted from the films. Quantification of crystal violet staining was performed for the section deletion mutants Ds4, Ds5, and Ds7. Δ50kb, WTdel, and biofilm former GspEΩ were analyzed as positive controls, while WT was analyzed as a negative control. For each data point, n ≥ 3.

5.2 The Biofilm Assay of the mutants from UGS Library

To further investigate individual genes for biofilm formation, we regenerated and confirmed segregated UGS mutants that possess individual insertional knockout mutations in a selected set of prophage genes. This set includes the genes composing sections 1 (Synpcc7942_0716, mutant 21C1) and section 6 (Synpcc7942_0754, mutant 21F6) in order to assay these gene sections. The subset of examined genes also includes all genes known to be transcriptionally or translationally expressed in WT according to Vijayan, et al. [2] and Guerrerio, et al. [3], respectively, as well as a number of genes of interest in the lysogeny and lysis gene sections. Whenever possible, two replicate clones were selected, confirmed, and quantitatively analyzed for biofilm formation. The data for each clone is shown in **Figure 5- 4**.

A number of clones displayed high levels of biofilm formation, such as 21E7 #1 and 21G3 #10. However, for the majority of these clones, the data were inconsistent with the alternative analyzed clone, such that while 21E7 #1 showed extremely high biofilm formation values, 21E7 #2 only showed moderate biofilm formation. To confirm these results, potential biofilm formers were selected and replicate experiments were again performed (**Figure 5- 5**). Of all the UGS mutants that have been tested to date, only four gene-inactivation mutants displayed consistent biofilm formation similar to the values observed with WTdel across multiple clones: the 21D10 mutation in the phage capsid protein gene Synpcc7942_0734, 21F10 in the gene Synpcc7942_0756 that encodes the phage lysozyme, 21F12 in Synpcc7942_0764 encoding a putative

transcriptional regulator likely maintaining lysogeny, and 21G3 in Synpcc7942_0766, which putatively encodes the phage antirepressor responsible inducing the prophage and is not expressed in WT. A number of mutants display biofilm formation values significantly higher than WT, but not consistently at levels similar to WTdel. Among these are mutants with clones displaying some of the highest biofilm values observed to date: 21C12 in Synpcc7942_0725, a putative DNA replication helicase that is not expressed in WT; 21E3 in a previously unannotated open reading frame that encodes a proline-alanine-alanine-arginine (PAAR) repeat protein that is homologous to the structural tip protein of the contractile phage spike or is often associated with the bacterial type VI secretion system; and 21E7 in Synpcc7942_0743, which is predicted to encode a virulence-associated tailspike protein with glycosyl-hydrolase activity. Interestingly, the last gene, Synpcc7942_0743, is not well expressed in WT, but an anti-sense transcript spanning the location of the 21E7 insertion is highly expressed in WT. This raises the possibility that the prophage element repressing biofilm forming is a non-coding RNA, rather than a protein.

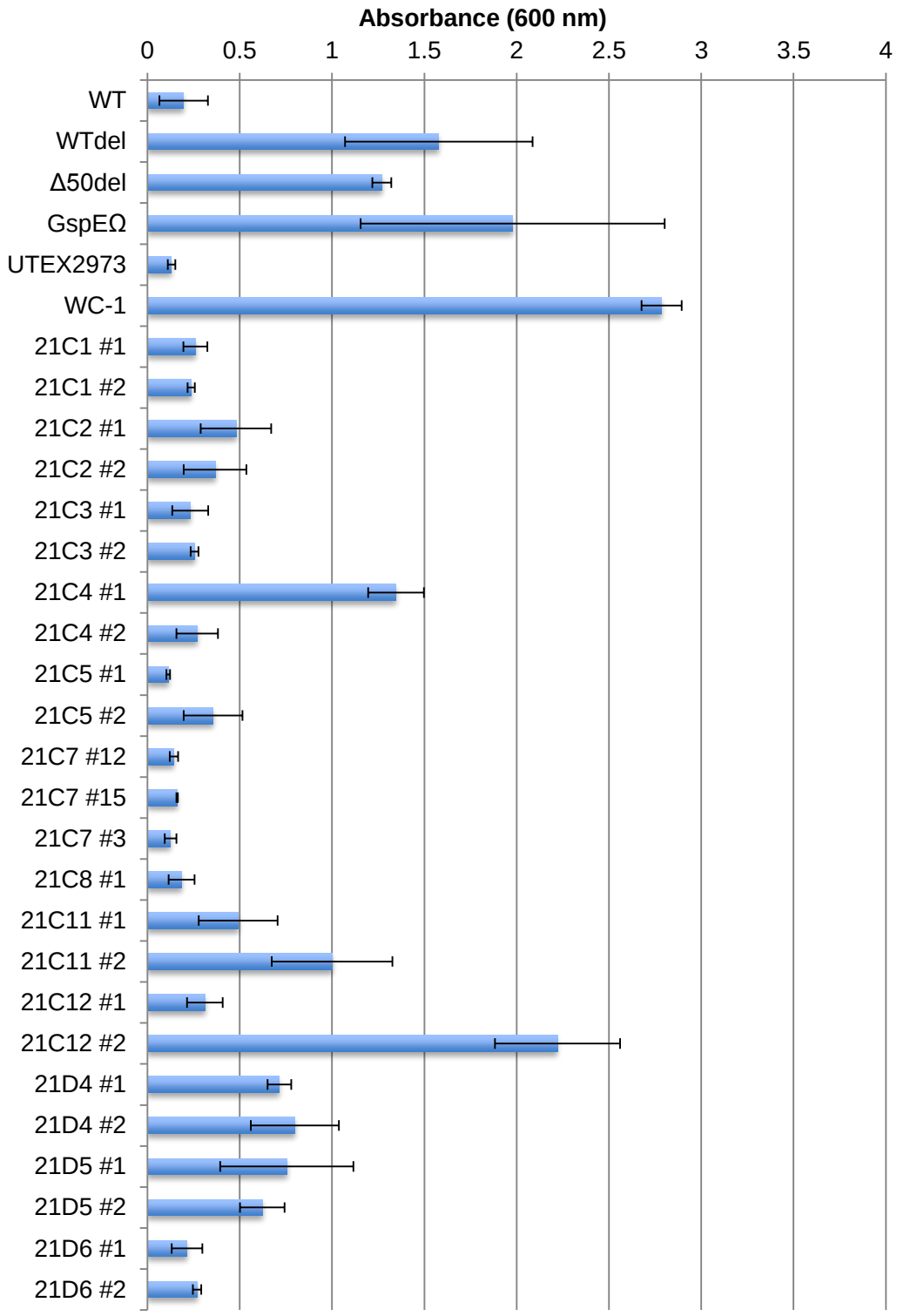

Figure 5- 4. UGS mutant biofilm assay. Biofilm analysis of individual UGS mutant clones, presented as in previous biofilm assay figures.

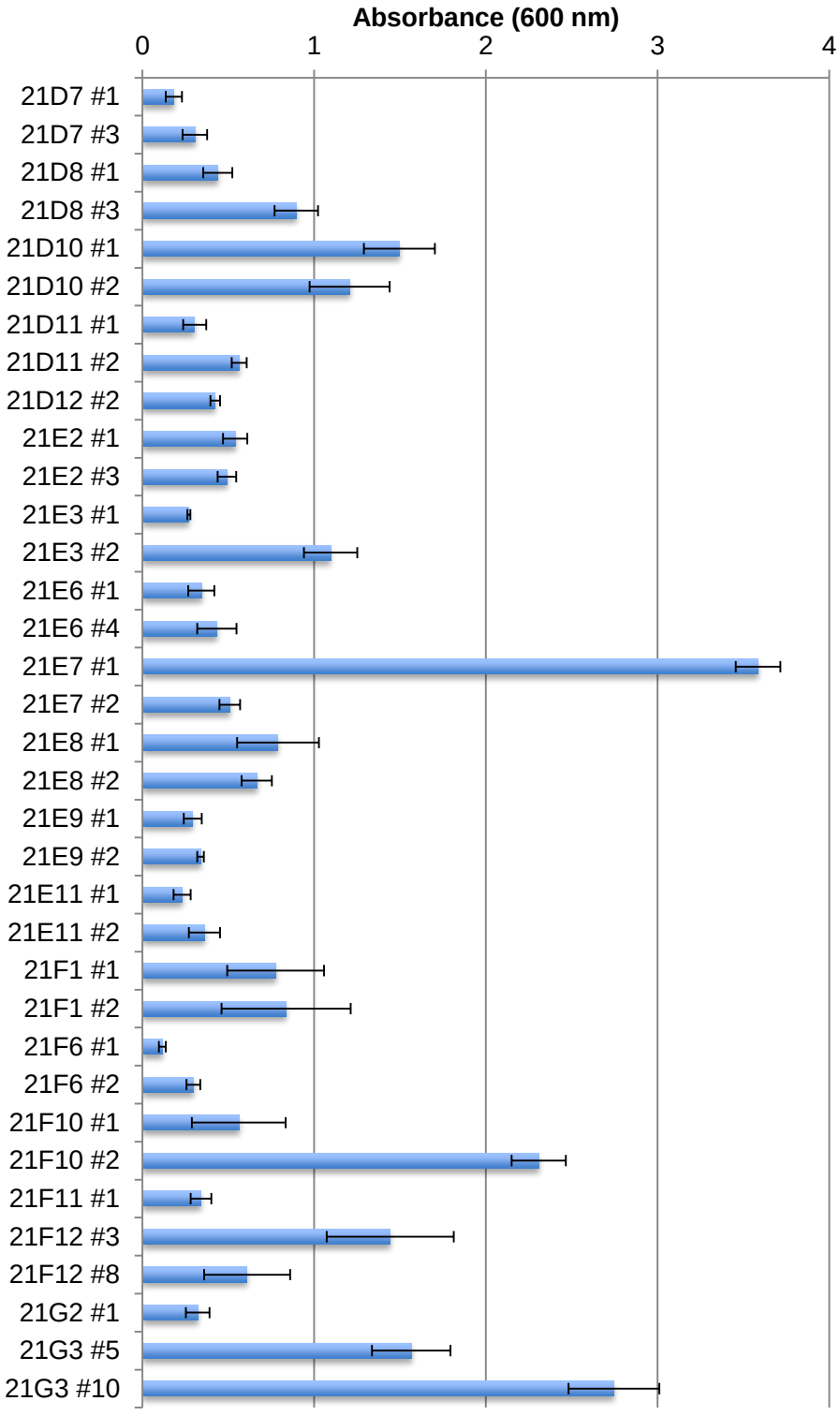

Figure 5- 4. UGS mutant biofilm assay, Continued. Biofilm analysis of individual UGS mutant clones, presented as in previous biofilm assay figures.

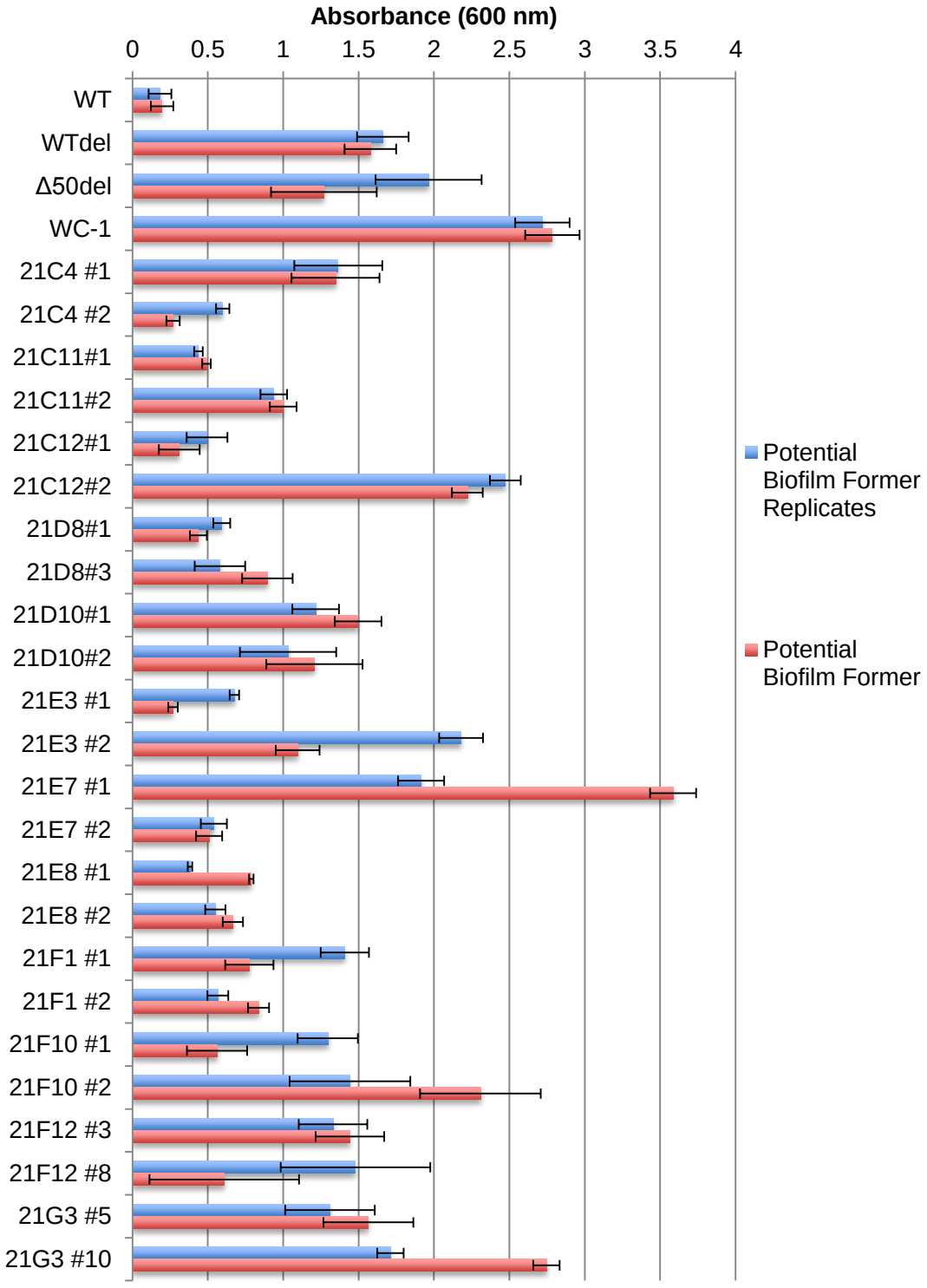

Figure 5- 5. Biofilm analysis of potential biofilm formers and replicate experiments. Data in red are as from Figure 5-5, while data in blue are from replicate experiments with the same clones. Biofilm analysis of individual UGS mutant clones, presented as in previous biofilm assay figures.

5.3 Deletions of Individual Genes and Biofilm Assay

Although the analysis of individual UGS gene mutants assisted in identifying potential repressors of the biofilm formation phenotype, a number of these gene candidates are located in the head and tail sections of the phage, sections 4 and 5 respectively, which do not display strong biofilm formation phenotypes when the entire section is deleted. This inconsistency led us to ask if the same observation would be made with mutations of genes in section 7. Only two of the six genes in section 7 have corresponding UGS mutations, with only one of those having been previously analyzed: 21F10 in the Synpcc7942_0756 lysozyme gene. The most likely explanation for the lack of transposon insertions in this region is a dramatic drop in the percent GC of the DNA sequence from approximately 60% to 35% following the tail section of the prophage, which retards transposon insertion. To examine more of the genes present in section 7, in particular the two genes that appear to be highly expressed in WT, SynpPCC7942_0759 and Synpcc7942_0760, I generated deletion vectors using the same strategy described for the section deletions. These vectors were then used to attempt deletions in WT.

I was able to successfully generate segregated deletion mutants in Synpcc7942_0757 (D0757), Synpcc7942_0759 (D0759), and Synpcc7942_0760 (D0760), although only a single segregated clone of D0757 was recovered and confirmed. All attempts to generate the deletion in Synpcc7942_0758 failed. I also generated deletions in the section 8 genes Synpcc7942_0761 (D0761) and Synpcc7942_0762 (D0762), which encode a

hypothetical protein and a putative helix-turn-helix transcriptional regulator, respectively. Although it was difficult to recover D0761 mutants, segregated clones were recovered.

Analyzing these deletion mutants for biofilm formation demonstrated that all of these mutants formed films to a degree significantly greater than WT, but significantly lower than WTdel, ∆50kb, and GspEΩ (**Figure 5- 6**). Unfortunately, these data do not enable any further insight as to whether an individual gene represses biofilm formation or a diversity of prophage genes contribute to this phenotype.

Given that a few potential biofilm repression gene candidates were identified, future experiments will focus on over-expressing these candidate genes or non-coding RNAs in biofilm-forming cells to determine their role in biofilm regulation.

Chapter 5, in full, is currently being prepared for submission for publication of the material. Simkovsky, Ryan; Golden, Susan. The thesis author was the primary investigator and author of this material.

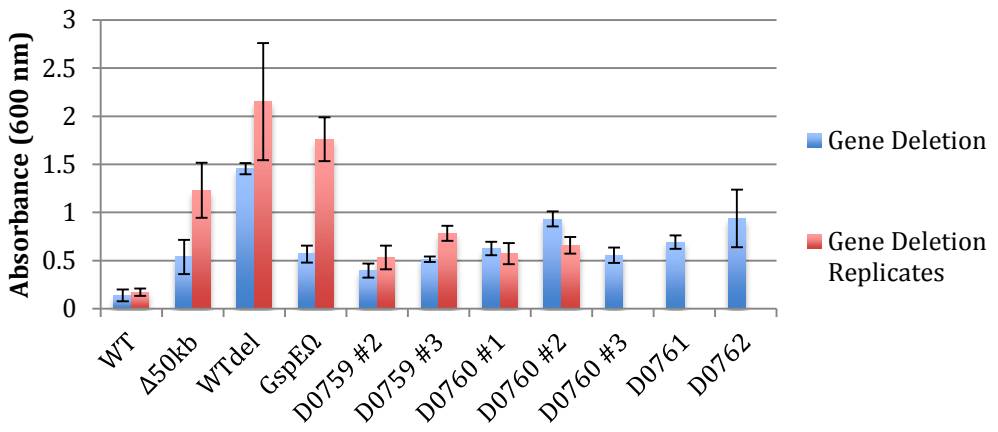

Figure 5- 6. Biofilm analysis of the deletion of individual genes in section 7 and section 8.

5.4 Reference

1.Rubin, B.E., K.M. Wetmore, M.N. Price, S. Diamond, R.K. Shultzaberger, L.C. Lowe, G. Curtin, A.P. Arkin, A. Deutschbauer, and S.S. Golden. "The essential gene set of a photosynthetic organism." *Proceedings of the National Academy of Sciences of the United States of America* 112, no. 48 (2015): E6634-6643. doi: 10.1073/pnas.1519220112

2.Vijayan, V., I.H. Jain, and E.K. O'Shea. "A high resolution map of a cyanobacterial transcriptome." *Genome biology* 12, no. 5 (2011): R47. doi: 10.1186/gb-2011-12-5-r47

3.Guerreiro, A.C.L., M. Benevento, R. Lehmann, B. van Breukelen, H. Post, P. Giansanti, A.F. Maarten Altelaar, I.M. Axmann, and A.J.R. Heck. "Daily rhythms in the cyanobacterium synechococcus elongatus probed by high-resolution mass spectrometry-based proteomics reveals a small defined set of cyclic proteins." *Molecular & cellular proteomics : MCP* 13, no. 8 (2014): 2042-2055. doi: 10.1074/mcp.M113.035840

Chapter 6

Discussion

6.1 The *S. elongatus* prophage represses its host's ability to form biofilms

We have demonstrated that the unicellular model cyanobacterium *Synechococcus elongatus* PCC 7942 is a lysogen – its genome carries a previously unrecognized prophage. The prophage genome appears to encode all of the genes necessary to regulate a temperate life cycle that is capable of switching between lytic and lysogenic states; excise, replicate, and package its genome; and build a tailed virion particle. This prophage is present in various forms in at least two other *S. elongatus* strains. Because of its existence in the genome of PCC 7942, the PCC 7942 prophage represents the first genetically manipulatable system for understanding the interaction between cyanophage and their cyanobacterial hosts.

In Chapter 3 of this thesis, I have demonstrated that genetic removal of the prophage uncovers its active role in regulating cyanobacterial physiology in living cells by quantitatively repressing biofilm formation. Through experiments beyond the scope of this thesis, Dr. Simkovsky, a postdoctoral researcher in the Golden lab, has demonstrated that removal of the prophage also increases the amount of photosynthetic pigments when grown under high light for long periods. Taken together, these findings first demonstrate that, in contrast to the

field's general view of prophage as inactive states in the phage life cycle, this prophage actively regulates host behaviors that are critical for its survival and adaptation to specific environments. Second, we hypothesize that these regulatory activities, likely occurring through expression of specific prophage encoded genes, determine both the ecological niche of the host and the environmental life cycle of the phage such that the phage infects a host adapted to low light levels in biofilms at the bottom of the water column and reprograms it to adapt to a light-intense, planktonic niche that allows for geographic dispersal.

In support of this hypothesis, we have demonstrated that the newly isolated strain WC-1 lacks the prophage, readily forms biofilms, and displays the increased photosystem pigmentation phenotype [1]. We are currently pursuing experiments to introduce the prophage into WC-1 to test for repression of these phenotypes in order to further support the environmental relevance of our findings.

We are also currently continuing our experiments to identify the genes responsible for biofilm repression and pigmentation regulation. As presented in Chapter 5, characterization of gene section deletions and individual gene knockouts or deletions has revealed a subset of candidate genes or non-coding RNAs that may repress biofilm formation. Deletions or knockouts of these candidate regulatory elements, however, have not produced strains that consistently produce biofilms to the same degree as has been observed when the prophage is absent from the cell. Further experiments using expression

constructs will be performed to verify the candidate biofilm repression genes or non-coding RNAs. Using these same deletion and knockout mutants, Dr. Simkovsky has narrowed down the regulators of the pigmentation phenotype to two genes expressed in the lysogen: Synpcc7942_0759 and/or Synpcc7942_0760 [1]. These data provide further evidence of the active role that the lysogen plays in manipulating its host and hope that a similarly small set of genes will be found that control the biofilm phenotype.

Based in part on these data and an ongoing collaboration with the laboratory of Dr. Rakefet Schwarz, we currently hypothesize that the regulation of biofilm formation may involve a complex network of regulatory factors and small RNAs that integrate information from the environment, from the state of the cell, and the prophage to determine biofilm formation. Dr. Schwarz's lab has determined that a lack of pili is a strong factor in enabling biofilm formation [2]. Based upon the results presented in Chapter 4 that mutations in *sigF* enhance the settling of *S. elongatus* and the fact that *sigF* regulates pili expression in PCC 6803, our findings would support the notion that SigF- or pili-regulated planktonicity is one of a number of factors repressing the biofilm state, but that the lack of either SigF or pili is not sufficient to activate biofilm formation. Interestingly, my data have also suggested a role for SigF in rescuing ΔrpaA LD sensitivity while Dr. Simkovsky has demonstrated that SigF does not contribute to the pigmentation phenotype [1].

In further support of the role of sigF and the pili in enhancing biofilm formation, I collaborated with PhD candidate Benjamin Rubin in the Golden lab

to screen a randomly barcoded transposon-insertional library [3] grown under biofilm-forming conditions in test tubes to identify genes that enhance or disable biofilm formation. High throughput sequencing of the barcoded library (RB-seq) and quantification of the relative population levels of every insertion in the biofilm versus the original culture resulted in a list of 68 genes that appear to repress biofilm formation and 35 genes that appear to enable inclusion or viability in the biofilm state. Included in the 68 repression genes are *sigF* and 14 pilin-encoding genes, again highlighting the regulatory role that these genes play in biofilm formation.

6.2 Technological applications of the PCC 7942 prophage

The prophage discussed in this thesis represents a powerful technology that can be applied to numerous industrial and environmental scenarios in order to actively alter the biofilm state of a cyanobacterial population for beneficial purposes. These include biomass-harvesting, production, prevention of biofouling and harmful algal sludge, and manufacturing of new biomaterials.

6.2.1 Enhancement of biofilms for biomass and bioproduct generation

Industrial growth of cyanobacteria for the purposes of generating biofuels, nutraceuticals, and other high-value products is predominantly performed in open pond systems, such as outdoor raceways. One major barrier to economic viability of these emerging markets is to reduce process costs, including the time, labor, and monetary expenses associated with

harvesting and dewatering the cyanobacterial crop. Our investigations suggest that removal of the prophage or inhibition of key biofilm-repressing elements encoded in the prophage will induce biofilm formation in *S. elongatus* populations. Formation of films can thus be exploited as an inducible mechanism for harvesting the planktonic cyanobacterial crop.

In contrast to the current planktonic growth-based practices, biofilm-based production systems have been proposed for algal biofuel and bioproduct generation [4]. These systems take advantage of the concentrated biomass inherent in biofilms to generate high product yields with minimal water and nutrient inputs. Additional benefits to these biofilm-based growth systems over the current planktonic growth systems include cost-efficient harvesting, reduced light limitation, and potential resistance to predators [5, 6] [7, 8]. Rotating algal biofilms (RABs), which combine the designs and benefits of planktonic open pond growth with biofilm-based growth on rotating surfaces, have proven to be among the most productive algal growth systems. These systems illustrate the technological potential of a cyanobacterial strain whose planktonic and biofilm growth behaviors can be readily manipulated by the addition or removal of the PCC 7942 prophage in order to optimize biomass production and harvesting in large-scale cyanobacterial growth systems.

Beyond mass production, many cyanobacterial biofilms can be beneficial to human health when employed in wastewater purification systems or bioremediation processes [9-12]. RABs were originally designed for wastewater purification purposes. It has also been demonstrated that some

phototrophic biofilms enhance degradation of some environmental toxins, such as microcystin [13]. The biofilm generating strains described in this thesis could enhance the generation of phototrophic, bioremediating communities.

6.2.2 Reduction of biofilms to prevent biofouling and harmful algal sludge

In both natural and industrial environments, the accumulation of microorganisms as scums or films on surfaces can lead to extensive problems for human health [14, 15] or maintenance of aquatic systems [10, 16]. Biofilms enable bacterial communities to withstand antibiotics, disinfectants, and predators, including the phagocytic cells of the innate immune system [17, 18]. These biofilm properties also protect cyanobacteria associated with harmful algal blooms, where these microorganisms produce toxins that can affect humans, livestock, and fish populations. Not only do these cyanobacterial films concentrate the harmful microbes, but they can also accumulate or concentrate the toxins in the scum present at the air-water interface, as is the case with microcystin produced by the cyanobacterium *Microcystis aeruginosa* [19] .

In all aquatic environments, accumulation of biofilms can lead to biofouling of artificial surfaces either in the form of disintegration of the material or in the excessive accumulation of substances to the point of impeding the function of the structure. For example, biofouling of naval vessels, which increases ship drag and reduces sailing efficiency, has been noted since the time of Plutarch [20].

Disruption of biofilms in all of the above scenarios is a desired goal and could be accomplished by using the PCC 7942 prophage as an anti-film agent. This may either be in the form of direct addition of the phage virion or through the application of key biofilm-repressing elements encoded in the prophage. Depending upon the ability to generate virion particles and the host range of the phage, the phage in theory could prevent film formation through either of two mechanisms: 1) lysis to disrupt the bacterial film community or 2) integration and repression of the bacterially-encoded biofilm formation pathway. Alternatively, upon identifying the genetic elements responsible for the biofilm repression phenotype, integration of expression constructs of the genes or application of the encoded elements could provide useful tools and technologies for biofilm disruption.

6.2.3 The prophage as a source for synthetic biology and biomanufacturing tools

In recent years, there has been a resurgence of interest in developing phage-based technologies and phage therapies [21]. Phage display or manipulation of viral components have been successfully developed to generate nanotechnology and novel bio-molecular materials, such as 3D printed nanofibers [22], antibacterial polymers [23], and biosensors [24-26]. Viral capsids have been engineered to function as imaging agents, including nanocomposite high-relaxivity MRI contrast agents [27], or conjugated to magnetic beads to act as sensors and antimicrobials against *E. coli* [28] or

Salmonella [29]. Most recently, manipulation of phage have allowed for the development of energy solutions in the form of energy generation [30] and biosolar panels [31]. Viruses, including bacteriophage, have been used to generate commercial bio-products widely [32, 33].

The *Synechococcus elongatus* PCC 7942 prophage is an advantageous platform for the development of similar phage-based technologies. In particular, the combination of the ease of genetic manipulation of the prophage in *S. elongatus* and the ability to grow it phototrophically to essentially convert solar energy directly into these various technologies makes the PCC 7942 prophage a very promising mass production platform. Given its natural freshwater environment, development of the prophage towards crop protection, harmful algal bloom control, and water waste remediation or monitoring are particularly interest [34].

6.3 Reference

1.Simkovsky, R., personal communication, 2015

2.Schwarz, R., personal communication, 2015

3.Rubin, B.E., K.M. Wetmore, M.N. Price, S. Diamond, R.K. Shultzaberger, L.C. Lowe, G. Curtin, A.P. Arkin, A. Deutschbauer, and S.S. Golden. "The essential gene set of a photosynthetic organism." *Proceedings of the National Academy of Sciences of the United States of America* 112, no. 48 (2015): E6634-6643. doi: 10.1073/pnas.1519220112

4.Gross, M., D. Jarboe, and Z. Wen. "Biofilm-based algal cultivation systems." *Applied microbiology and biotechnology* 99, no. 14 (2015): 5781-5789. doi: 10.1007/s00253-015-6736-5

5.DePas, W.H., A.K. Syed, M. Sifuentes, J.S. Lee, D. Warshaw, V. Saggar, G. Csankovszki, B.R. Boles, and M.R. Chapman. "Biofilm Formation Protects Escherichia coli against Killing by Caenorhabditis elegans and Myxococcus xanthus." *Applied and Environmental Microbiology* 80, no. 22 (2014): 7079-7087. doi: 10.1128/aem.02464-14

6.Halan, B., K. Buehler, and A. Schmid. "Biofilms as living catalysts in continuous chemical syntheses." *Trends in biotechnology* 30, no. 9 (2012): 453-465. doi: 10.1016/j.tibtech.2012.05.003

7.Wang, Z.-W. and S. Chen. "Potential of biofilm-based biofuel production." *Applied microbiology and biotechnology* 83, no. 1 (2009): 1-18. doi: 10.1007/s00253-009-1940-9

8.Christenson, L.B. and R.C. Sims. "Rotating algal biofilm reactor and spool harvester for wastewater treatment with biofuels by-products." *Biotechnology and bioengineering* 109, no. 7 (2012): 1674-1684. doi: 10.1002/bit.24451

9.Egan, S., T. Thomas, and S. Kjelleberg. "Unlocking the diversity and biotechnological potential of marine surface associated microbial communities." *Current Opinion in Microbiology* 11, no. 3 (2008): 219-225. doi: 10.1016/j.mib.2008.04.001

10.Ivnitsky, H., I. Katz, D. Minz, G. Volvovic, E. Shimoni, E. Kesselman, R. Semiat, and C.G. Dosoretz. "Bacterial community composition and structure of biofilms developing on nanofiltration membranes applied to wastewater treatment." *Water research* 41, no. 17 (2007): 3924-3935. doi: 10.1016/j.watres.2007.05.021

11.Roeselers, G., M.C.M.v. Loosdrecht, and G. Muyzer. "Phototrophic biofilms and their potential applications." *Journal of Applied Phycology* 20, no. 3 (2008): 227-235. doi: 10.1007/s10811-007-9223-2

12.Wagner, M. and A. Loy. "Bacterial community composition and function in sewage treatment systems." *Current opinion in biotechnology* 13, no. 3 (2002): 218-227. doi: 10.1016/S0958-1669(02)00315-4

13.Babica, P., L. Bláha, and B. Marsálek. "Removal of microcystins by phototrophic biofilms. A microcosm study." *Environmental science and pollution research international* 12, no. 6 (2005): 369-374

14.Costerton, J.W., P.S. Stewart, and E.P. Greenberg. "Bacterial biofilms: a common cause of persistent infections." *Science (New York, N.Y.)* 284, no. 5418 (1999): 1318-1322

15.Costerton, J.W., K.J. Cheng, G.G. Geesey, T.I. Ladd, J.C. Nickel, M. Dasgupta, and T.J. Marrie. "Bacterial biofilms in nature and disease." *Annu Rev Microbiol* 41, (1987): 435-464. doi: 10.1146/annurev.mi.41.100187.002251

16.Flemming, H.C., G. Schaule, T. Griebe, J. Schmitt, and A. Tamachkiarowa. "Biofouling—the Achilles heel of membrane processes." *Desalination* 113, no. 2–3 (1997): 215-225. doi: 10.1016/S0011-9164(97)00132-X

17.Joo, H.S. and M. Otto. "Molecular basis of in vivo biofilm formation by bacterial pathogens." *Chem Biol* 19, no. 12 (2012): 1503-1513. doi: 10.1016/j.chembiol.2012.10.022

18.Mah, T.F. and G.A. O'Toole. "Mechanisms of biofilm resistance to antimicrobial agents." *Trends Microbiol* 9, no. 1 (2001): 34-39

19.Butler, N., J.C. Carlisle, R. Linville, and B. Washburn. "Microcystins: A Brief Overview of their Toxicity and Effects, with Special Reference to fish,

Wildlife and Livestock." (2009): 1-21

20.Plutarch. "Essays and Miscellanies." *The Complete Works Volume 3.* Last modified February 7, 2013. http://www.gutenberg.org/ebooks/3052?msg=welcome_stranger

21.Kutter, E.M., S.J. Kuhl, and S.T. Abedon. "Re-establishing a place for phage therapy in western medicine." *Future microbiology* 10, no. 5 (2015): 685-688. doi: 10.2217/fmb.15.28

22.Wang, J., M. Yang, Y. Zhu, L. Wang, A.P. Tomsia, and C. Mao. "Phage Nanofibers Induce Vascularized Osteogenesis in 3D Printed Bone Scaffolds." *Advanced Materials* 26, no. 29 (2014): 4961-4966. doi: 10.1002/adma.201400154

23.Mao, J.Y., A.M. Belcher, and K.J. Van Vliet. "Antibacterial Coatings: Genetically Engineered Phage Fibers and Coatings for Antibacterial Applications (Adv. Funct. Mater. 2/2010)." *Advanced Functional Materials* 20, no. 2 (2010): n/a-n/a. doi: 10.1002/adfm.201090001

24.Lee, J.-W., J. Song, M.P. Hwang, and K.H. Lee. "Nanoscale bacteriophage biosensors beyond phage display." *International journal of nanomedicine* 8, no. 1 (2013): 3917-3925. doi: 10.2147/IJN.S51894

25.Farr, R., D.S. Choi, and S.-W. Lee. "Phage-based nanomaterials for biomedical applications." *Acta Biomaterialia* 10, no. 4 (2014): 1741-1750. doi: 10.1016/j.actbio.2013.06.037

26.Wu, Y., S. Feng, X. Zan, Y. Lin, and Q. Wang. "Aligned Electroactive TMV Nanofibers as Enabling Scaffold for Neural Tissue Engineering." *Biomacromolecules* 16, no. 11 (2015): 3466-3472. doi: 10.1021/acs.biomac.5b00884

27.Qazi, S., L.O. Liepold, M.J. Abedin, B. Johnson, P. Prevelige, J.A. Frank, and T. Douglas. "P22 viral capsids as nanocomposite high-relaxivity MRI contrast agents." *Molecular pharmaceutics* 10, no. 1 (2013): 11-17. doi: 10.1021/mp300208g

28.Chen, J., S.D. Alcaine, Z. Jiang, V.M. Rotello, and S.R. Nugen. "Detection of Escherichia coli in drinking water using T7 bacteriophage-conjugated

magnetic probe." *Analytical chemistry* 87, no. 17 (2015): 8977-8984. doi: 10.1021/acs.analchem.5b02175

29.Fernandes, E., V.C. Martins, C. Nóbrega, C.M. Carvalho, F.A. Cardoso, S. Cardoso, J. Dias, D. Deng, L.D. Kluskens, P.P. Freitas, and J. Azeredo. "A bacteriophage detection tool for viability assessment of Salmonella cells." *Biosensors and Bioelectronics* 52, (2014): 239-246. doi: 10.1016/j.bios.2013.08.053

30.Lee, B.Y., J. Zhang, C. Zueger, W.-J. Chung, S.Y. Yoo, E. Wang, J. Meyer, R. Ramesh, and S.-W. Lee. "Virus-based piezoelectric energy generation." *Nat Nano* 7, no. 6 (2012): 351-356. doi: 10.1038/nnano.2012.69

31.Vilona, D., R. Di Lorenzo, M. Carraro, G. Licini, L. Trainotti, and M. Bonchio. "Viral nano-hybrids for innovative energy conversion and storage schemes." *J. Mater. Chem. B* 3, no. 33 (2015): 6718-6730. doi: 10.1039/C5TB00924C

32.Hefferon, K.L. "Plant virus expression vectors set the stage as production platforms for biopharmaceutical proteins." *Virology* 433, no. 1 (2012): 1-6. doi: 10.1016/j.virol.2012.06.012

33.Vicente, T., J.P.B. Mota, C. Peixoto, P.M. Alves, and M.J.T. Carrondo. "Rational design and optimization of downstream processes of virus particles for biopharmaceutical applications: current advances." *Biotechnology advances* 29, no. 6 (2011): 869-878. doi: 10.1016/j.biotechadv.2011.07.004

34.Jofre, J., A.R. Blanch, F. Lucena, and M. Muniesa. "Bacteriophages infecting Bacteroides as a marker for microbial source tracking." *Water research* 55, (2014): 1-11. doi: 10.1016/j.watres.2014.02.006

Appendix

Materials and Methods

Generating tagging and deletion vectors

Tagging and deletion vectors were constructed using the GeneArt® Seamless Cloning and Assembly Kit (Life Technologies). Assembly DNA devices were derived from CYANO-VECTOR donor plasmids using restriction enzymes *Zra*I or *Eco*RV-HF (New England BioLabs) followed by DNA purification and concentration with DNA Clean & Concentrator™- 5 kit (Zymo), as detailed in Taton, et al [1]. Assembly amplicons were generated by PCR using Q5 polymerase (New England BioLabs), as per the manufacturers instructions, and verified through gel electrophoresis on 0.7% agarose gels. Prior to seamless assembly, amplicons greater than 300 bp were purified with the Invitrogen PureLink® PCR Purification Kit (Life Technologies) using the High-Cutoff Binding Buffer (B3) to remove primer dimers and small, non-specific PCR products.

Assembled vectors were transformed into chemically competent DH5α or One Shot® TOP10 (Life Technologies) *E. coli* according to standard heat shock procedures or the manufacturer's instructions. Transformed *E. coli* were grown at 37 °C overnight on solid LB agar plates containing 50 µg/ml kanamycin (Km) or 20 µg/ml spectinomycin (Sm) and 20 µg/ml streptomycin (Sp), dependent upon the vector design, to select for cells harboring the

desired vector. Colonies were counter-screened for ampicillin-resistance through replica-plating onto solid LB agar plates containing 100 µg/ml ampicillin (Ap). Clones lacking Ap-resistance but continuing to grow on the desired antibiotics were inoculated into 5 ml liquid cultures and processed for DNA via the QIAprep Spin Miniprep Kit (Qiagen) or ethanol precipitation. All vectors were confirmed by PCRs designed to amplify across assembly junctions, restriction enzyme digest analysis designed to produce unique band patterns in the product versus parental donor vectors, and sequencing of recombination regions and junctions.

Cosmids for generating transposon-insertional knockout mutants into individual gene were obtained from the unigene set (UGS) library [2, 3]. Knockouts of the *sigF* gene, *Synpcc7942_1510*, were obtained from private library cosmids 4E8-N5 (referred to as N5) and 1E9-D7 (referred to as D7).

Generating mutants of *Synechococcus elongatus* PCC 7942

Confirmed deletion, tagging, or UGS vectors were transformed into fresh cultures of *S. elongatus* PCC 7942 that were grown at 150 µmol photons $m^{-2}s^{-1}$ at 30 °C. Transformation was performed according to standard methods that take advantage of *S. elongatus*' natural competence and ability to perform double homologous recombination [4-6]. Transformants were selected on solid BG-11 media [7] supplemented with kanamycin at 5 µg/mL, spectinomycin at 2 µg/mL, streptomycin at 2 µg/mL, or 5% sucrose, as appropriate for the desired strain. Cultures derived from transformation colonies were grown in BG-11

media either in liquid or on solid medium containing the appropriate antibiotics under constant light levels ranging from 100 – 200 µmol photons $m^{-2}s^{-1}$ at 30 °C. Liquid cultures were analyzed for bacterial or fungal contamination regularly using BG-11 Omni medium: solid BG-11 agar media supplemented with 0.04% (wt/vol) glucose and 5% (vol/vol) LB.

Transformants were analyzed for double homologous recombination and complete conversion or segregation of all chromosomal copies via PCR analysis designed to amplify the locus of interest. In all cases, the amplicon generated from the WT locus differed significantly in size from that derived from the mutant locus so that gel electrophoresis analysis of the PCR products would differentiate WT from mutant loci. Confirmed clones were grown and maintained as described above.

Quantitative assays for biofilm formation

In order to assay strains for biofilm formation in a quantitative and high-throughput manner, a number of methodologies for preparing biofilms in culture were attempted. A substantial amount of time and effort was spent testing and optimizing these protocols due to the requirement to have a reproducible and quantitative assay to analyze and compare the large number of strains generated for this project. Therefore, the important lessons and protocol points will be described here.

In preparation for the biofilm formation assays, lawns of cultures were first grown on BG-11 plates containing the appropriate antibiotics. This

solid-medium starter culture is a necessary step for the biofilm assay so as to eliminate or infinitely dilute any accumulated biofilm repressor that would inhibit subsequent film formation [8]. Scrapings of those lawns were then added to starter cultures in 250 ml flasks containing 15 ml of BG-11R medium without antibiotics and incubated for four days on an orbital shaker at 30°C under constant light intensity of ~150 µmol photons $m^{-2}s^{-1}$. The BG-11R medium contains freshly made iron solutions and 4% HEPES as a buffer, as per the biofilm formation media preparation detailed in Schatz, et al [9]. The growth of the starter culture in a low volume of medium in a 250 ml flask was important for obtaining high densities of cell cultures in a short period of time, so as to prevent accumulation of the secreted biofilm repressor. Presumably, the rapid growth and increased cell densities were due to the combination of decreased shading effects and increased gas exchange due to the high surface area to volume ratio. It was also important to acclimate the cultures to BG11R media at this stage, prior to the initiation of the biofilm formation assay.

As stated, biofilm formation in cultures was tested using several different culturing methods. These methods include the published bubbling tube protocol of our collaborators in the Schwarz lab [9], multiple multi-well plate protocols, and biofilm formation in stationary flasks, which is the condition under which the Golden lab originally observed biofilms when initiating the collaboration with the Schwarz lab. Due to the fact that the bubbling tube assay, as performed in our lab, is not particularly high-throughput and films often fell off the curved, glass surface during post-formation washing steps, numerous

multi-well plate protocol were tested for biofilm formation. Variations to the well plates included differing material compositions (polystyrene vs. poly-D-lysine vs. glass), surface treatments (poly-D-lysine coated vs. Corning Cell Bind Surface vs. Ultra Low Attachment Surface vs. BioCoat for attachment purpose vs. non-coated), well sizes and numbers (from 6 well plates to 96 well plates), and well-bottom shapes (flat vs. conical vs. curved). Plates were also tested under different conditions, including varying temperature (room temperature or 30 °C) and light conditions (~3 to 150 µmol photons $m^{-2}s^{-1}$). In our hands, no single plate or condition proved to perform consistently or significantly when assaying our control strains: WT, T2SEΩ obtained from the Schwarz lab, and the ∆50kb strain.

A modified multi-well plate assay was tested based upon advice from the Curtiss lab [10] was attempted in which glass coverslips were placed in the bottom of 6-well plate wells. The notion behind this protocol is that the glass would improve biofilm attachment and also enable observation of the films under the microscope. However, not all control strains formed films under these conditions and those biofilms that did appear simply fell off the coverslip upon washing.

Of the methodologies examined and optimized, only the small flask cultures resulted in significant and consistent data across positive and negative biofilm formation controls. All quantitative biofilm formation values reported in this thesis were performed using the following flask-based biofilm-formation assay.

To perform the biofilm assay in small flask cultures, starter cultures were diluted to an OD at 750 nm of 0.5 using fresh BG11-R media and 5 ml aliquots of this dilution were placed in sterile 25 ml flasks plugged with cotton balls. Due to potential build up salts or other residues that can impact biofilm formation, these flasks were autoclaved with water present in the flask, as opposed to a dry autoclave cycle, prior to removal of the water and subsequent addition of medium and culture. Although biofilms appear to form better in the bottoms of old vs new flasks, the biofilms formed on scratched vs. non-scratched clean flasks were compared, which gave no significant difference. Most of the flasks labeled as flat bottom have a slightly curved bottom due to manufacture issue, but only the flasks with real flat bottoms supported stronger biofilms. To maintain the same condition for every experiment, all biofilm formation data in this thesis were performed in new 25 ml Pyrex flasks (catalog number 4980). Biofilm formation flasks were incubated at low light, approximately 5 µmol photons $m^{-2}s^{-1}$, at room temperature without shaking for 7 days.

Biofilm formation was quantified according to a modified version of the crystal violet staining technique of Fisher, et al. [10]. In brief, liquid cultures were removed by decanting and the culture flasks were gently washed with water three times and air-dried prior to staining with 5 ml of 1% crystal violet in each flask for 15 min. Stain that remained in the biofilm after three additional water washes was extracted by a 5 ml of modified biofilm dissolving solution (MBDS) containing 10% sodium dodecyl sulfate (SDS) dissolved in 80% ethanol. The volume of the crystal violet stain and MBDS were same as the

volume of the culture to stain all biofilms, no matter whether the culture was grown in flasks or tubes. A 200-µl volume of each extract solution was transferred to 96-well plates and measured by absorbance spectroscopy at 600 nm using an Infinite® M200 absorbance plate reader (Tecan) to quantify the amount of crystal violet that was bound to the biofilm. For each experimental strain, multiple biological replicates were assayed.

LD-sensitivity spot assay

To test for the LD-sensitivity phenotype previously described for the deletion of the *rpaA* gene in the WT *S. elongatus* PCC 7942 background [11], exponentially growing cultures were diluted to an optical density at 750 nm of 0.494. These dilutions were subsequently serially diluted 1:5 five times. 4 µl of each serial dilution was spotted onto BG-11 solid media. Serial dilution cultures plates were incubated at 30°C with 12:12 artificial light:dark cycles or constant light at 149 µmol photons $m^{-2}s^{-1}$ for 2 weeks and checked for growth of the cultures.

Reference

1.Taton, A., F. Unglaub, N.E. Wright, W.Y. Zeng, J. Paz-Yepes, B. Brahamsha, B. Palenik, T.C. Peterson, F. Haerizadeh, S.S. Golden, and J.W. Golden. "Broad-host-range vector system for synthetic biology and biotechnology in cyanobacteria." *Nucleic Acids Research* 42, no. 17 (2014): e136-e136. doi: 10.1093/nar/gku673

2.Chen, Y., C.K. Holtman, A. Taton, and S.S. Golden. "Functional analysis of the synechococcus elongatus PCC 7942 Genome." In *Advances in Photosynthesis and Respiration*, edited by R.L. Burnap and W.F.J. Vermaas. p. 119-137. Springer Science+Business Media B.V. . 2012

3.Holtman, C.K., Y. Chen, P. Sandoval, A. Gonzales, M.S. Nalty, T.L. Thomas, P. Youderian, and S.S. Golden. "High-throughput functional analysis of the Synechococcus elongatus PCC 7942 genome." *DNA research : an international journal for rapid publication of reports on genes and genomes* 12, no. 2 (2005): 103-115. doi: 10.1093/dnares/12.2.103

4.Clerico, E.M., J.L. Ditty, and S.S. Golden. "Specialized techniques for site-directed mutagenesis in cyanobacteria." *Methods in molecular biology (Clifton, N.J.)* 362, no. 11 (2007): 155-171. doi: 10.1007/978-1-59745-257-1_11

5.Golden, S.S., J. Brusslan, and R. Haselkorn. "Genetic engineering of the cyanobacterial chromosome." *Methods in enzymology* 153, (1987): 215-231

6.Golden, S.S. and L.A. Sherman. "Optimal conditions for genetic transformation of the cyanobacterium Anacystis nidulans R2." *Journal of Bacteriology* 158, no. 1 (1984): 36-42

7.Allen, M.M. "Simple Conditions for Growth of Unicellular Blue-Green Algae on Plates 1, 2." *Journal of Phycology* 4, no. 1 (1968): 1-4. doi: 10.1111/j.1529-8817.1968.tb04667.x

8.Schwarz, R., personal communication, 2014

9.Schatz, D., E. Nagar, E. Sendersky, R. Parnasa, S. Zilberman, S. Carmeli, Y.

Mastai, E. Shimoni, E. Klein, O. Yeger, Z. Reich, and R. Schwarz. "Self-suppression of biofilm formation in the cyanobacterium Synechococcus elongatus." *Environmental Microbiology* 15, no. 6 (2013): 1786-1794. doi: 10.1111/1462-2920.12070

10. Fisher, M.L., R. Allen, Y. Luo, and R. Curtiss. "Export of extracellular polysaccharides modulates adherence of the Cyanobacterium synechocystis." *PloS one* 8, no. 9 (2013): e74514. doi: 10.1371/journal.pone.0074514

11. Takai, N., M. Nakajima, T. Oyama, R. Kito, C. Sugita, M. Sugita, T. Kondo, and H. Iwasaki. "A KaiC-associating SasA-RpaA two-component regulatory system as a major circadian timing mediator in cyanobacteria." *Proceedings of the National Academy of Sciences of the United States of America* 103, no. 32 (2006): 12109-12114. doi: 10.1073/pnas.0602955103