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Bioinformatic Comparison and Salinity Tolerance of Various Cyanobacterial Strains

Lucie Rowe

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BIOINFORMATIC COMPARISON AND SALINITY TOLERANCE OF VARIOUS CYANOBACTERIAL STRAINS

By
Lucie Beatrice Rowe

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College

Oxford, MS
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Approved By

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Advisor: Professor Paul Boudreau

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Reader: Professor Saumen Chakraborty

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Reader: Professor Brenton Laing
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ABSTRACT

LUCIE BEATRICE ROWE: Bioinformatic Comparison and Salinity Tolerance of Various Cyanobacterial Strains
(Under the direction of Dr. Paul Boudreau)

Cyanobacteria are an abundant and diverse group of photosynthetic prokaryotes with a long evolutionary history, which presents challenges for comprehensive taxonomic classification. Phylogenies were traditionally constructed based on the highly conserved 16S rRNA gene, but research has increasingly relied upon whole genome sequencing to elucidate evolutionary relationships, despite increased cost and time. In this thesis, publicly available genome sequences of various cyanobacterial strains were utilized to determine if the whole rRNA region, which includes the 16S, 23S, and 5S genes as well as the spacer regions between, could provide accurate, yet cost/time efficient representations of their evolutionary relationships. From phylogenetic trees constructed using the Geneious Prime® software as well as TYGS, it was determined that an approach using the whole rRNA region remained concordant with 16S data, and therefore was not uniquely informative.

Recognizing the importance of a polyphasic approach, applying classical taxonomy in addition to genomics, this thesis also assessed the salinity tolerance of Boudreau Lab cyanobacterial strains from various freshwater sources in northern Mississippi. Salinity tolerance of cyanobacteria is not only significant for characterizing novel strains, but is also of increasing importance to understand fundamental cyanobacterial biology and its impact on algal bloom formation, an environmental and
human threat which has become more prevalent due to climate change and pollution in aquatic environments. From culture inoculations into media prepared over various salt concentrations, it was determined that the majority of Boudreau Lab cyanobacterial strains are able to tolerate some degree of salinization to their environment.

In future work, DNA isolation and genome sequencing of the Boudreau Lab isolates would be necessary to build a phylogeny for our strain library. Salinity tolerance assays for the remaining strains in the Boudreau Lab collection should also be performed, so the upper limit of growth under elevated salt conditions is known for all isolates. Genomic study of the Boudreau Lab collection could also inform any possible genetic basis of salinity tolerance for our isolates by identifying shared genetic information among strains which displayed similar salinity tolerance results.
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<th>Description</th>
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<tr>
<td>ALE</td>
<td>Adaptive Laboratory Evolution</td>
</tr>
<tr>
<td>DFAST™</td>
<td>DDBJ Fast Annotation and Submission Tool</td>
</tr>
<tr>
<td>DSMZ</td>
<td>German Collection of Microorganisms and Cell Cultures</td>
</tr>
<tr>
<td>ITS</td>
<td>Internal Transcribed Spacer</td>
</tr>
<tr>
<td>MUSCLE</td>
<td>Multiple Sequence Comparison by Log-Expectation</td>
</tr>
<tr>
<td>TYGS</td>
<td>Type Strain Genome Server</td>
</tr>
</tbody>
</table>
CHAPTER ONE: COMPARISON OF BIOINFORMATIC APPROACHES FOR STUDYING CYANOBACTERIAL EVOLUTION

INTRODUCTION

As an extremely diverse and abundant clade of photosynthetic prokaryotes which evolved roughly 3.5 billion years ago, cyanobacteria possess vital ecological roles, contributing to both the carbon and nitrogen cycles (Willis & Woodhouse, 2020). They are the only organisms to have evolved oxygenic photosynthesis; other organisms gained this ability through endosymbiosis with cyanobacteria, for example, the chloroplast organelle in true plants (Schirrmeister, et al., 2015). Through the action of cyanobacterial photosynthesis producing oxygen, Earth’s atmosphere became oxygenated in the early Proterozoic, triggering the development of far more complex lifeforms on Earth (Schirrmeister, et al., 2015). However, creating a comprehensive taxonomy of these organisms proves challenging because of their long evolutionary history, high morphological and ecological diversity, and instances of homologous recombination and horizontal gene transfer (Willis & Woodhouse, 2020).

Commonly, genetic comparison of the highly conserved 16S rRNA gene, an approach developed by Carl Woese in the 1990s, has been used to determine phylogenetic relationships among cyanobacteria (Woese, 1990). However, studies from as early as 2001 began to question the efficacy of using the 16S rRNA region alone,
especially when considering species-level distinctions among genera, and instead suggest a longer portion of the DNA strand, such as the 16S-23S rRNA internal transcribed spacer (ITS) region, as being potentially more accurate for genetic comparison (Boyer, et al., 2001). In the last two decades, whole genome studies have been shown to provide a comprehensive approach to studying the differences among cyanobacterial strains, however this method proves to be a rather time consuming process due to the more sensitive DNA isolation protocols and expensive sequencing methods required (Leão, et al., 2017).

Considering these limitations of the various methods for elucidating phylogenetic relationships among cyanobacteria, the aim of this chapter is to explore if whole rRNA region analyses improve on the limitations of 16S data. This approach could still rely on the cost/time saving benefits of avoiding whole genome sequencing. As part of this effort, publicly available genome sequences of various cyanobacterial strains will be used to determine if phylogenetic trees built with the whole rRNA region, which includes the 16S, 23S, and 5S genes as well as the spacer regions between, can provide accurate representations of evolutionary relationships among cyanobacterial strains.

MATERIALS AND METHODS

General Experimental Methods

Phylogenetic comparison of the cyanobacteria was conducted using the Geneious Prime® 2022.0 software platform as well as the Type (Strain) Genome Server, TYGS, available through the Leibniz Institute DSMZ, the German Collection of Microorganisms
and Cell Cultures (www.geneious.com) (Meier-Kolthoff & Göker, 2019). Genomes of cyanobacterial strains used in this research were obtained from GenBank®, a National Institutes of Health database for all publicly available DNA sequences (Benson, et al., 2018). Additionally, DFAST™, the DDBJ Fast Annotation and Submission Tool, available from the DNA Data Bank of Japan, National Institute of Genetics, was used for gene annotation when needed (Tanizawa, et al., 2018).

**Downloading Genomes from GenBank**

On GenBank®, the genome search tool was utilized to search for strains with filters set to output only annotated genomes with an assembly level of chromosome or higher. Genome sequences and their annotations were downloaded from GenBank® as GBFF files and imported into the Geneious Prime® software for construction of ribosomal gene phylogenetic trees. For each cyanobacterial strain, its accession number and year of publication were noted. It is important to recognize that occasionally the same strain is uploaded to GenBank® more than once, as different versions under multiple accession numbers due to corrections, or resequencing, of the original strain. In the case that multiple files of the same strain were available for download, the most recent year or most complete genome sequence was selected for use in this thesis.

**Annotating Genomes**

While annotated genomes of cyanobacterial strains were preferred, there were several instances where GenBank® genome sequences did not include annotations for a specific strain of interest. To annotate these strains, the genome sequence was imported into the Geneious Prime® software, then exported as a FASTA file to the DFAST™ web
server. Once annotations of the genome were completed on DFAST™, the annotated file was reuploaded to Geneious Prime® to be used in building phylogenetic trees.

Construction of Whole rRNA Region Phylogenetic Trees

On Geneious Prime®, the annotated genome sequence of interest was filtered for rRNA regions. The whole rRNA region (16S, 23S, and 5S) was then selected and extracted from the annotated genome sequence of the strain and labeled “rRNA region 1.” Some strains possessed two or three rRNA regions in their genome sequence. These additional rRNA regions were also extracted and labeled as rRNA region(s) 2 and/or 3, respectively. For alignment purposes, the reverse complement tool on Geneious Prime® was used to change the directionality of rRNA region strands when necessary so all strands were read in the same relative direction for the ribosomal genes, as is shown in Figure 1-1.

**Figure 1-1**: Annotated rRNA Regions in Geneious Prime® Software

![Annotated rRNA Regions](image)

The annotated rRNA regions 1 and 2 of *Synechococcus* sp. CC9605 (GenBank® accession number CP000110) were extracted from the complete genome, where the reverse complement tool was used to ensure the same relative directionality for strains possessing multiple ribosomal regions in their genome.

These sequences were first aligned on the Geneious Prime® software with a MUSCLE, Multiple Sequence Comparison by Log-Expectation, alignment using 8 iterations. The final phylogenetic trees of these MUSCLE alignments were run using the Geneious Tree Builder Tool, with the Jukes-Cantor Genetic Distance Model and the Neighbor-Joining Tree Build Method. No outgroup was selected, allowing for pairwise distances to be
obtained from the multi sequence alignment. Resampling of trees was performed using bootstraps with 50,000 replicates, with a support threshold of 50%.

Construction of 16S rRNA Region and Whole Genome Trees

To construct the 16S rRNA and whole genome trees of cyanobacterial strains, the GenBank® accession numbers noted in the construction of the whole rRNA region phylogenetic tree on Geneious Prime® were copied and pasted into the TYGS web server. Restricting the data set to query genomes, the consensus trees constructed by TYGS contained the same strains as the tree constructed through Geneious Prime®. Both 16S rRNA gene and whole genome phylogenetic trees were prepared by TYGS server.

RESULTS AND DISCUSSION

For the first set of phylogenetic trees, genome sequences from the genus *Synechococcus* were selected from GenBank® because this genus is ubiquitous in nearly all habitats (Dvořák et al., 2014). Further, strains in this genus are commonly used as a robust model cyanobacterium in many research fields (Zhang et al., 2024). From the available *Synechococcus* strains on GenBank®, 16 genome sequences were downloaded and then phylogenetic trees were constructed in accordance with the Materials and Methods for this chapter. The final trees for these 16 strains of *Synechococcus*, which will be referred to as Dataset 1, are shown in Figures A-1, A-2, and A-3 in Appendix A. The grouping of strains in the 16S versus whole genome tree were distinct, as would be expected, given previous research which indicates whole genome studies as more comprehensive in comparison to what can be accomplished with 16S data alone (Leão, et
al., 2017). When comparing the 16S TYGS tree to the whole rRNA region tree we created, the strains had nearly identical groupings, suggesting that whole rRNA region phylogenetic trees are not uniquely informative. However, we were concerned that this small sample size was insufficient, and therefore chose to improve upon these initial results by including more representatives in a second comparison.

Trees were expanded from Dataset 1 to a total of 30 strains by including additional *Synechococcus* strains as well as several *Thermosynechococcus* strains, which were anticipated to appear as a separate clade (Salazar, et al., 2020). The final trees for Dataset 2 are shown in Figures 1-2, 1-3, and 1-4. As was seen in Dataset 1, the grouping of strains in the 16S tree versus the whole genome tree remained distinct. Notably, *Thermosynechococcus* was grouped monophyletically in the 16S tree, but not in the whole genome tree. In the whole rRNA region tree created on Geneious Prime®, *Thermosynechococcus* appeared as a monophyletic group and all other strains were group similarly to the 16S data, again indicating that whole rRNA region phylogenetic trees do not provide unique insights into the evolutionary relationships of cyanobacterial strains over the 16S analysis.
**Figure 1-2:** 16S Region Phylogenetic Tree for Dataset 2

![Phylogenetic tree diagram](image)

**Figure 1-2** is the 16S phylogenetic tree created using TYGS for Dataset 2, which includes cyanobacterial 30 strains from *Synechococcus* and *Thermosynechococcus*. 
**Figure 1-3:** Whole Genome Phylogenetic Tree for Dataset 2

![Phylogenetic Tree Diagram]

**Figure 1-3** is the whole genome phylogenetic tree created using TYGS for Dataset 2, which includes 30 cyanobacterial strains from *Synechococcus* and *Thermosynechococcus*. 
**Figure 1-4**: Whole rRNA Region Phylogenetic Tree for Dataset 2

Figure 1-4 shows the whole rRNA region phylogenetic tree (displayed horizontally) that was created on Geneious Prime® for Dataset 2. Geneious Prime® trees display the same strain name multiple times, due to the presence of multiple ribosomal regions for that specific strain.
Lastly, we were curious to see whether these results would remain consistent if a greater diversity of strains, comprising more diverse genera, were used in the analysis. Therefore, for Dataset 3, 18 strains available for download on GenBank® from *Synechococcus, Prochlorococcus, Pleurocapsales, Oscillatoriales, Nostocales*, and *Chroococcales* were utilized. The final phylogenetic trees of Dataset 3, created according to the Materials and Methods for this chapter, are shown in **Figures 1-5, 1-6, and 1-7** on the next several pages. In Dataset 3, the grouping of strains in the 16S tree versus the whole genome tree were again distinct. Additionally, the whole rRNA region tree created on Geneious Prime® grouped strains similarly to the 16S tree, reinforcing the idea that whole rRNA region analyses do not likely make major improvements on 16S data.
**Figure 1-5**: 16S Region Phylogenetic Tree for Dataset 3

Figure 1-5 is the 16S phylogenetic tree created using TYGS for Dataset 3, which includes a diversity of strains from multiple cyanobacteria genera.
**Figure 1-6**: Whole Genome Phylogenetic Tree for Dataset 3

![Phylogenetic Tree Diagram](image)

- *Synechococcus elongatus* PCC 6301' (NZ_CP085785)
- *Synechococcus elongatus* UTEX 2973' (NZ_CP006471)
- *Microcystis aeruginosa* NIES-2549' (CP011304)
- *Anabaena cylindrica* PCC 7122' (CP003659)
- *Prochlorococcus* sp. MIT 0801' (CP007754)
- *Stanieria cyanosphaera* PCC 7437' (CP003653)
- *Calothrix* sp. PCC 6303' (CP003610)
- *Pleurocapsa* sp. PCC 7327' (CP003590)
- *Synechococcus* sp. JA-3-3A' (CP000239)
- *Allocolepsis franciscana* PCC 7113' (CP003630)
- *Prochlorococcus marinus* subsp. marinus str. CCMP1375' (AE017126)
- *Geitlerinema* sp. PCC 7407' (CP003591)
- *Gloeothecae verrucosa* PCC 7822' (CP0002198)
- *Nostoc azollae* 0706' (CP002059)
- *Moorena producens* PAL-8-15-08-1' (CP017599)
- *Halothece* sp. PCC 7418' (CP003945)
- *Prochlorococcus marinus* str. AS9601' (CP000551)
- *Synechococcus* sp. OC9605' (CP000110)

**Figure 1-6** is the whole genome phylogenetic tree created using TYGS for Dataset 3, which includes a diversity of strains from multiple cyanobacteria genera.
**Figure 1-7:** Whole rRNA Region Phylogenetic Tree for Dataset 3

![Phylogenetic Tree](image)

**Figure 1-7** is the whole rRNA region tree for Dataset 3, which includes a diversity of strains from multiple cyanobacteria genera. This phylogenetic tree was created using the Geneious Prime® software. This tree displays the same strain name multiple times, due to the presence of multiple ribosomal regions for that specific strain.
CONCLUSION AND FUTURE DIRECTIONS

Overall, from the phylogenetic trees created with Geneious Prime® and TYGS using various cyanobacterial strains, whole rRNA region analyses, which includes the 16S, 23S, and 5S genes as well as the spacer regions between, does not seem to improve on the limitations of 16S data while still providing the cost/time saving benefits of avoiding whole genome sequencing. Fortunately, there is ongoing research for developing new, more efficient protocols for DNA isolation and sequencing methods for cyanobacteria that would allow whole genome analyses to become more accessible (Wagner, 2023).

For the future directions of this research, it may be helpful to create additional phylogenetic trees using whole rRNA region analyses as confirmation of these results, perhaps including a much larger sample size, or other cyanobacterial genera. Additionally, these findings could be applied to ongoing projects in the Boudreau Lab involving the DNA isolation and genome sequencing of over 50 unidentified strains from various freshwater sources in northern Mississippi.
CHAPTER TWO: SALINITY TOLERANCE OF BOUDREAU LAB

CYANOBACTERIAL STRAINS

INTRODUCTION

While the prior chapter of this thesis emphasizes the value of genetic comparison for elucidating the phylogenetic relationships among cyanobacterial strains, historical approaches to studying evolutionary history, such as those relying on morphological, physiological, and ecological data, should not be entirely cast aside. Research in these areas can still provide insights which may be helpful for distinguishing between genera as well as characterizing novel strains, recent studies argue for utilizing a polyphasic approach, applying both classical taxonomy and genomics, to the study of these organisms (Cordeiro et al., 2020)(Mogany et al., 2018).

Given that cyanobacteria are known to occupy a wide range of ecological niches with varied chemistries, from desert soil, to caves, freshwater streams, brackish estuaries, marine environments, and even thermal lakes, their salinity tolerance is an area of considerable interest (Cordeiro et al., 2020)(Jung et al., 2022). There are many mechanisms by which cyanobacteria resist salt stress, some of which are even species-specific (Cui, et al., 2020). One such method is the accumulation of organic osmolytes, highly soluble organic compounds with low molecular mass, for the maintenance of osmotic potential and turgor pressure (Hagemann, 2011).
Additionally, many cyanobacteria utilize transporters, such as Na⁺/H⁺ antiporters, Mrp systems, Ktr systems, and/or Na⁺-pumping ATPases to maintain ionic homeostasis, exporting Na⁺ and Cl⁻ out of their cells when in brackish and saltwater environments (Hagemann, 2011)(Cui, et al., 2020). Some cyanobacteria under salt stress even exhibit increased accumulation of unsaturated fatty acids, which could be an adaptive response to maintain membrane fluidity, given the lower melting point of unsaturated fatty acids relative to saturated fatty acids (Cui, et al., 2020).

Climate change, pollution from deicing salts and mining operations, and eutrophication due to agricultural runoff have also altered the chemistry of many of the environments in which cyanobacteria exist (Hintz, et al., 2022). These changes in the environment have exacerbated the incidence of cyanobacterial algal blooms, which are known to release toxins harmful to vertebrates, including humans (Chorus et al., 2021). While mechanical and biological strategies, such as sediment dredging and the addition of filter-feeding organisms, are currently being explored to prevent the growth of algal blooms from drinking water sources, these methods have cost limitations and potential negative environmental impacts (Kibuye, et al., 2021). Further, current governmental water quality guidelines are not sufficient in protecting our freshwater sources from pollutants and salinization (Hintz, et al., 2022). Thus, studies of salinity tolerance among cyanobacteria strains are important in order to understand fundamental cyanobacterial biology and its impact on bloom formation, an environmental and human threat. With this in mind, this chapter of this thesis will specifically focus on salinity tolerance of cyanobacterial strains in the Boudreau Lab collection, which primarily consists of isolates from freshwater sources in northern Mississippi.
MATERIALS AND METHODS

General Experimental Equipment and Materials

Media at various salinities was prepared by the addition of solid sodium chloride (Fisher Science Education™, Lab Grade) to the freshwater BG-11 media which was prepared according to a recipe modified from the University of Texas at Austin Culture Collection of Algae (see Appendix B). The MilliQ water used in media preparation was obtained from the MilliQ water system provided by the University of Mississippi Center of Biomedical Research Excellence in Natural Products Neuroscience. Prepared media was then sterilized in an autoclave, a Steris™ AMSCO® C Series Remanufactured Small Steam Sterilizer. Over 50 strains grown in continuous liquid culture in the Boudreau Lab were available for use. These strains were previously isolated by members of the Boudreau Lab from an orchid plant root as well as various freshwater sources in northern Mississippi, including Enid, Sardis, Puskus, Lamar Bruce, Grenada, and Arkabutla Lakes and the Mississippi River (Wagner, 2023). Cultures were grown under 1550 lux on a 16:8 light/dark cycle timer using Gardener’s Supply Company™ LED grow lights.

Media Preparation

For all media, roughly 800 mL of MilliQ water was first added to a 2.8 L Erlenmeyer flask. Then, 10 mL of BG-11 Stocks 1-7 and 1 mL of BG-11 Stock 8 were added to the 2.8 L Erlenmeyer flask according to the freshwater BG-11 media recipe (see Appendix B). After the addition of each stock, the solution was thoroughly mixed. Importantly, Stock 5 was maintained under sterile conditions to prevent bacterial growth in this stock. Once all the stocks were added, the solution was diluted to 1000 mL using
MilliQ water. To the 2.8 L Erlenmeyer flask, an appropriate amount of NaCl was added to achieve the desired salinity. Notably, the freshwater BG-11 media is natively approximately 1.7 ppt salt (see Appendix B). Although, for clarity, all salinity values given in this thesis reference only the amount of added NaCl to the baseline salinity of freshwater BG-11 media. For example, to prepare media which will be defined as having a salinity of 3.5 ppt, 3.5 g of NaCl was added to a 1 L preparation of freshwater BG-11 media. Once salt was added, 50 mL volumes of the media were transferred into 125 mL Erlenmeyer flasks. Each flask was covered with a cotton ball and aluminum foil and labeled before being autoclaved on a 15 minute liquids cycle.

**Culture Inoculation**

Into each autoclaved 125 mL Erlenmeyer flask containing 50 mL of freshwater BG-11 media, with or without added NaCl, a Boudreau Lab cyanobacteria strain was passaged under sterile conditions. Of the available strains, 38 were randomly selected to be included in the preliminary experiment due to space constraints. From these 38 strains utilized in the preliminary experiment, some of which were further investigated over a wider range of salinities in subsequent experimentation. Sterile conditions were maintained by spraying down the lab bench and equipment with a 70% ethanol in water solution before working under a flame using sterile pipette tips. Passages were performed using 1 μL/mL inoculation, where 50 μL from the live Boudreau Lab liquid culture of a strain was transferred by pipette into each flask containing 50 mL of media. Inoculations were performed in triplicate and each experiment had a control group of freshwater BG-11 media.
Observation of Growth

Cultures were left under the grow lights and monitored in the Boudreau Lab for roughly two months following inoculation. The visual observations of growth were recorded, as variable morphology and mat formation made traditional OD measurement unreliable. For the purposes of this experiment, cultures were observed as exhibiting no growth, limited growth, or normal growth relative to the control freshwater BG-11 media. Normal growth was assigned to cultures at a salt concentration where there were clear indications of growth including color changes of the previously clear media to green and/or observable green particulates, scums, foams, or mats similar to the freshwater controls. On the other hand, no growth was defined as cultures at salt levels without indications of the presence of chlorophyll, as assessed by a lack of observable green particulates, scums, foams, or mats in the media. Limited growth was given relative to the control, where less biomass, or change in color, such as yellowing of the cyanobacterial filaments, was noted.

RESULTS AND DISCUSSION

Considering that all Boudreau Lab strains utilized for this experiment were collected from freshwater sources, a total of 38 were grown in freshwater BG-11 media with 3.5 ppt NaCl in triplicate as a baseline for salt tolerance. Previous studies have noted the addition of NaCl as an appropriate surrogate for seawater in salt tolerance assays for cyanobacteria, as NaCl exerts a similar or higher toxicity than seawater (Venâncio, et al., 2018). Average salinity of seawater is approximately 35 ppt (Pawłowicz, 2013).
However, we did not expect that these freshwater strains would be able to tolerate the salt levels of seawater conditions. Therefore, a tenfold decrease in concentration, to 3.5 ppt NaCl, was used for this baseline test because it models salt concentration above their native environments but still far below seawater. The results of this preliminary experiment for each strain can be seen in Table A-1 included in Appendix C.

Of the 38 strains tested, over 80% exhibited either growth or limited growth at this salinity value, suggesting that most strains in the Boudreau Lab collection can tolerate some degree of salinization to their environment. Additionally, all strains sourced from the Mississippi River as well as the orchid plant root grew well at 3.5 ppt NaCl. On the other hand, strains sourced from several lakes in northern Mississippi had more variability in their salt tolerance. For example, various strains from Sardis Lake and Grenada Lake exhibited growth, limited growth, or no growth at a concentration of 3.5 ppt NaCl.

Using the results of this preliminary experiment, some of the Boudreau Lab strains were tested over various ranges of salt concentrations to determine their maximum salt tolerance. For strains exhibiting no growth in the baseline experiment, all six were tested over a range of 0 to 4.0 ppt added NaCl, to find their salinity tolerance below 3.5 ppt. For this range, Boudreau Lab strains were grown in triplicate at 0, 1.0, 2.0, 3.0 and 4.0 ppt NaCl. From the seven strains with limited growth in the baseline experiment, six were randomly selected to be tested over a range of 3.0 to 10.5 ppt NaCl to ensure robust experimental conditions, seeing that they likely could tolerate salinization only slightly above 3.5 ppt NaCl. In this range, strains were grown in triplicate at 0, 3.0, 5.0, 7.5, and 10.5 ppt NaCl. For strains exhibiting normal growth in the baseline experiment, five were
tested at a range from 3.5 - 70.0 ppt NaCl, evaluating the growth of these strains up to salt concentrations representative of hypersaline environments such as brine pools (Bougouffâ, et al., 2013). Within this range, strains were grown in triplicate at 0, 3.5, 5.0, 7.0, 15.0, 25.0, 35.0, 45.0, and 70.0 ppt NaCl. For these three subsequent salinity tolerance tests, Table 2-1 displays strains and their respective upper limit of growth as determined over the aforementioned salinity ranges.

**Table 2-1: Salinity Tolerance over Various Ranges of Selected Boudreau Lab Strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Salinity Range Tested</th>
<th>Upper Limit of Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL-A-07</td>
<td>Enid Lake water</td>
<td>0 - 4.0 ppt NaCl</td>
<td>2.0 ppt NaCl</td>
</tr>
<tr>
<td>BL-A-12</td>
<td>Sardis Lake water</td>
<td>0 - 4.0 ppt NaCl</td>
<td>2.0 ppt NaCl</td>
</tr>
<tr>
<td>BL-A-15</td>
<td>Lamar Bruce Lake water</td>
<td>0 - 4.0 ppt NaCl</td>
<td>2.0 ppt NaCl</td>
</tr>
<tr>
<td>BL-A-20</td>
<td>Enid Lake water</td>
<td>0 - 4.0 ppt NaCl</td>
<td>1.0 ppt NaCl</td>
</tr>
<tr>
<td>BL-A-34</td>
<td>Grenada Lake water</td>
<td>0 - 4.0 ppt NaCl</td>
<td>3.0 ppt NaCl</td>
</tr>
<tr>
<td>BL-A-51</td>
<td>Arkabutla Lake water</td>
<td>0 - 4.0 ppt NaCl</td>
<td>0 ppt NaCl</td>
</tr>
<tr>
<td>BL-A-02</td>
<td>Grenada Lake water</td>
<td>3.0 - 10.5 ppt NaCl</td>
<td>3.0 ppt NaCl</td>
</tr>
<tr>
<td>BL-A-03</td>
<td>Enid Lake water</td>
<td>3.0 - 10.5 ppt NaCl</td>
<td>5.0 ppt NaCl</td>
</tr>
<tr>
<td>BL-A-09</td>
<td>Grenada Lake water</td>
<td>3.0 - 10.5 ppt NaCl</td>
<td>5.0 ppt NaCl</td>
</tr>
<tr>
<td>BL-A-11</td>
<td>Enid Lake water</td>
<td>3.0 - 10.5 ppt NaCl</td>
<td>7.5 ppt NaCl</td>
</tr>
<tr>
<td>BL-A-13</td>
<td>Enid Lake water</td>
<td>3.0 - 10.5 ppt NaCl</td>
<td>7.5 ppt NaCl</td>
</tr>
<tr>
<td>BL-A-25</td>
<td>Sardis Lake water</td>
<td>3.0 - 10.5 ppt NaCl</td>
<td>0 ppt NaCl</td>
</tr>
<tr>
<td>BL-A-04</td>
<td>Grenada Lake water</td>
<td>3.5- 70.0 ppt NaCl</td>
<td>5.0 ppt NaCl</td>
</tr>
<tr>
<td>BL-A-08</td>
<td>Sardis Lake water</td>
<td>3.5- 70.0 ppt NaCl</td>
<td>7.0 ppt NaCl</td>
</tr>
<tr>
<td>BL-A-10</td>
<td>Orchid plant root</td>
<td>3.5- 70.0 ppt NaCl</td>
<td>35.0 ppt NaCl</td>
</tr>
<tr>
<td>BL-A-17</td>
<td>Puskus Lake water</td>
<td>3.5- 70.0 ppt NaCl</td>
<td>7.0 ppt NaCl</td>
</tr>
<tr>
<td>BL-A-44</td>
<td>Grenada Lake water</td>
<td>3.5- 70.0 ppt NaCl</td>
<td>5.0 ppt NaCl</td>
</tr>
</tbody>
</table>

Table 2-1 displays the upper limit of growth over various salinity ranges tested for selected Boudreau Lab strains, where the salinity range tested was determined from the results of the initial baseline test (see Table A-1) and the upper limit of growth determined visually, relative to the control freshwater BG-11 media and other salinity values tested, as is explained in the methods for Chapter Two.
Interestingly, over the various salinity ranges tested, some strains’ growth appeared as a gradient, with progressive decreases in the biomass or relative lightening of color. On the other hand, some strains had a distinct limit of growth, where the strain grew very well relative to the control, until a salinity with a distinct lack of growth. These observations are exemplified in Figures A-4 and A-5 included in Appendix C.

Among the strains tested, only one strain, BL-A-10, exhibited growth at 35.0 ppt NaCl, the average salinity value of seawater. Further, no strains grew in the 45.0 ppt NaCl nor the 70.0 ppt NaCl media, thus cultures at 70.0 ppt NaCl were not informative for finding the upper limit of growth of freshwater Boudreau Lab strains. Aside from BL-A-10, the upper limit of growth was 7.5 ppt NaCl or lower for all other strains. Due to the limited laboratory space, perhaps including more values at lower salt ranges in lieu of growing cultures at 70.0 ppt NaCl would better serve future experimentation. For example, instead of growing strains in the 70.0 ppt NaCl media, they could be grown at 10.0 ppt NaCl.

Although BL-A-25 showed limited growth in the preliminary experiment at 3.5 ppt NaCl, this strain only grew in the control freshwater BG-11 media in subsequent experimentation. Given that all other strains tested in this range exhibited growth, it is unlikely that there was an issue with the media itself. Rather, BL-A-25 was likely inoculated improperly in either the preliminary or subsequent experiment, leading to conflicting results of its salinity tolerance. This strain should be retested in order to determine its upper limit of growth.

From these results, starting with a salinity value of 3.5 ppt NaCl could be subject to change in future experimentation for the baseline test because, as seen in Table A-1 in
Appendix C, the majority of strains still grew in the 3.5 ppt NaCl media. Likewise, as shown in **Table 2-1** for the results of subsequent experimentation, both the median value and mode for the upper limit of growth was 5.0 ppt NaCl, indicating that a salt concentration of 5.0 ppt NaCl may be more suitable for dividing the freshwater Boudreau Lab strains into groups for salinity tolerance testing.

**CONCLUSION AND FUTURE DIRECTIONS**

In summary, from these salinity tolerance experiments, the upper limit of growth for many of the Boudreau Lab strains from various freshwater sources in Mississippi was determined, which is vital information for their characterization, most especially in the case that some isolates included in this thesis are novel strains, as is suggested by previous work by the Boudreau Lab (Wagner, 2023).

For the future directions of this research, it would first be necessary to perform salinity tolerance studies for the remaining strains in the Boudreau Lab collection so the upper limit of growth under elevated salt conditions is known for all isolates. Genomic study of the Boudreau Lab collection could be used to explain any possible genetic basis of salinity tolerance among these freshwater Mississippi isolates by identifying shared genetic information among strains which displayed similar salinity tolerance results. Additionally, Adaptive Laboratory Evolution (ALE) technique, gradually increasing the salt stress in order to cultivate halotolerant strains, could be applied to selected strains (Cui, et al., 2020). ALE could help us to understand the specific mechanisms by which
Boudreau Lab isolates resist salt stress through comparison of parent cells and salt-adapted cells (Hu, et al., 2020).

Overall, Chapter Two of this thesis highlights the importance of the polyphasic approach to studying evolutionary relationships among organisms, where salinity tolerance studies not only provided previously unknown information on Boudreau Lab strains, but also could serve as a foundation for future bioinformatic work.
BIBLIOGRAPHY


APPENDICES

A. SUPPLEMENTAL PHYLOGENETIC TREES

Figure A-1: 16S Region Phylogenetic Tree for Dataset 1

Figure A-1 shows the phylogenetic tree created on TYGS using 16S data for Dataset 1, which includes 16 unique Synechococcus strains. There are two instances where the same strain has been uploaded twice, under different accession numbers.
Figure A-2: Whole Genome Phylogenetic Tree for Dataset 1

Figure A-2 shows the phylogenetic tree created on TYGS using whole genome data for Dataset 1, which includes 16 unique Synechococcus strains. There are two instances where the same strain has been uploaded twice, under different accession numbers.
Figure A-3 displays the phylogenetic tree created on Geneious Prime® using the whole rRNA region(s) extracted from the genome sequences of 16 unique Synechococcus strains included as Dataset 1. Geneious Prime® trees display the same strain name multiple times, due to the presence of multiple ribosomal regions for that specific strain.
B. CYANOBACTERIAL MEDIA

Freshwater BG-11 Media

Protocol obtained from the University of Texas at Austin Culture Collection of Algae:
https://utex.org/products/bg-11-medium?variant=30991786868826#recipe

Note the modification to mix stocks 5 and 6 together.

Components:

1. NaNO₃ Stock (30 g / 200 mL)................................. 10 mL/ L
2. K₂HPO₄ Stock (0.8 g / 200 mL)............................... 10 mL/ L
3. MgSO₄ 7H₂O (1.5 g / 200 mL)................................. 10 mL/ L
4. CaCl₂ 2H₂O (0.72 g / 200 mL)............................... 10 mL/ L
5. Citric Acid Monohydrate (0.12 g / 200 mL) **AND**
   Ferric Ammonium Citrate (0.12 g / 200 mL)........... 10 mL/ L
6. Na₂EDTA 2H₂O (0.02 g/200 mL).............................. 10 mL/ L
7. Na₂CO₃ (0.40 g / 200 mL).................................... 10 mL/ L
8. BG-11 Trace Metals Solution................................. 1 mL/ L
9. Sodium thiosulfate pentahydrate (49.6 g / 200 mL)*... 1 mL/ L

* [ONLY FOR AGAR PLATES]
8. Trace Metals Solution:

- H$_2$BO$_3$ [Boric acid]................................................................. 2.86 g / L
- MnCl$_2$ 4H$_2$O [Manganese (III) chloride, tetrahydrate].................. 1.81 g/ L
- ZnSO$_4$ 7H$_2$O [Zinc sulfate, heptahydrate]................................. 0.22 g/ L
- Na$_2$MoO$_4$ 2H$_2$O [Sodium molybdate, dihydrate]....................... 0.39 g/ L
- CuSO$_4$ 5H$_2$O [Copper (II) sulfate, pentahydrate]....................... 0.079 g/ L
- Co(NO$_3$)$_2$ 6H$_2$O [Cobalt (II) nitrate, hexahydrate].................... 0.0494 g/ L

Protocol:

1. Add components in order to ~900 mL of MilliQ while stirring
2. If making agar plate, add 15 g of agar
3. Dilute to 1 L and autoclave
### C. SUPPLEMENTAL TABLES AND FIGURES FOR CHAPTER TWO

#### Table A-1: Baseline Salinity Tolerance of Boudreau Lab Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Growth at 3.5 ppt NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL-A-01</td>
<td>Orchid plant root</td>
<td>Normal growth</td>
</tr>
<tr>
<td>BL-A-02</td>
<td>Grenada Lake water</td>
<td>Limited growth</td>
</tr>
<tr>
<td>BL-A-03</td>
<td>Enid Lake water</td>
<td>Limited growth</td>
</tr>
<tr>
<td>BL-A-04</td>
<td>Grenada Lake water</td>
<td>Normal growth</td>
</tr>
<tr>
<td>BL-A-06</td>
<td>Sardis Lake water</td>
<td>Normal growth</td>
</tr>
<tr>
<td>BL-A-07</td>
<td>Enid Lake water</td>
<td>No growth</td>
</tr>
<tr>
<td>BL-A-08</td>
<td>Sardis Lake water</td>
<td>Normal growth</td>
</tr>
<tr>
<td>BL-A-09</td>
<td>Grenada Lake water</td>
<td>Limited growth</td>
</tr>
<tr>
<td>BL-A-10</td>
<td>Orchid plant root</td>
<td>Normal growth</td>
</tr>
<tr>
<td>BL-A-11</td>
<td>Enid Lake water</td>
<td>Limited growth</td>
</tr>
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<td>BL-A-12</td>
<td>Sardis Lake water</td>
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</tr>
<tr>
<td>BL-A-13</td>
<td>Enid Lake water</td>
<td>Limited growth</td>
</tr>
<tr>
<td>BL-A-15</td>
<td>Lamar Bruce Lake water</td>
<td>No Growth</td>
</tr>
<tr>
<td>BL-A-17</td>
<td>Puskus Lake water</td>
<td>Normal growth</td>
</tr>
<tr>
<td>BL-A-19</td>
<td>Puskus Lake water</td>
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</tr>
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<td>BL-A-20</td>
<td>Enid Lake water</td>
<td>No growth</td>
</tr>
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<td>BL-A-25</td>
<td>Sardis Lake water</td>
<td>Limited growth</td>
</tr>
<tr>
<td>BL-A-26</td>
<td>Arkabutla Lake water</td>
<td>Normal growth</td>
</tr>
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<td>BL-A-27</td>
<td>Grenada Lake water</td>
<td>Limited growth</td>
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<td>BL-A-29</td>
<td>Arkabutla Lake water</td>
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<tr>
<td>BL-A-43</td>
<td>Sardis Lake water</td>
<td>Normal growth</td>
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</table>
Table A-1 (Continued)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Growth at 3.5 ppt NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL-A-44</td>
<td>Grenada Lake water</td>
<td>Normal growth</td>
</tr>
<tr>
<td>BL-A-45</td>
<td>Mississippi River</td>
<td>Normal growth</td>
</tr>
<tr>
<td>BL-A-46</td>
<td>Arkabutla Lake water</td>
<td>Normal growth</td>
</tr>
<tr>
<td>BL-A-47</td>
<td>Grenada Lake water</td>
<td>Normal growth</td>
</tr>
<tr>
<td>BL-A-48</td>
<td>Arkabutla Lake water</td>
<td>Normal growth</td>
</tr>
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<td>BL-A-49</td>
<td>Mississippi River</td>
<td>Normal growth</td>
</tr>
<tr>
<td>BL-A-50</td>
<td>Sardis Lake water</td>
<td>Normal growth</td>
</tr>
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<td>BL-A-51</td>
<td>Arkabutla Lake water</td>
<td>No growth</td>
</tr>
<tr>
<td>BL-A-53</td>
<td>Mississippi River</td>
<td>Normal growth</td>
</tr>
</tbody>
</table>

This table displays the results of a baseline salinity tolerance test, where Boudreau Lab Strains were grown in Freshwater BG-11 Media with 3.5 ppt NaCl added. Observations of growth were recorded visually, according to the Methods for Chapter Two.

Figure A-4: Gradient Growth of BL-A-13

Figure A-4 shows BL-A-13 grown in triplicate in the 3.0 - 10.5 ppt salt test group (left to right) after roughly two months. As discussed in the Results and Discussion section of Chapter Two, some Boudreau Lab strains had progressive decreases in growth with increased NaCl concentration over their tested range, creating a visual gradient of relative color and observable particulates, scums, foams, or mats. The upper limit of growth was estimated to be 5.0 ppt NaCl, based on the relatively limited growth in values above this salinity.
Figure A-5: Distinct Loss of Growth in BL-A-15

Figure A-5 shows BL-A-13 grown in triplicate in the 0 - 4.0 ppt salt test group (right to left) after roughly two months, with a distinct loss of growth over the test range. Visually, growth remained relatively unchanged until reaching 3.0 ppt NaCl, where the media now appeared essentially clear. Thus, the upper limit of growth was determined to be 2.0 ppt NaCl.