Spring 5-7-2022

A Comparison of Heterotroph Isolation and Sequencing Methods From Various Cyanobacterial and Algal Microbiomes

Victoria Starks

A COMPARISON OF HETEROTROPH ISOLATION AND SEQUENCING METHODS FROM VARIOUS CYANOBACTERIAL AND ALGAL MICROBIOMES

By

Victoria Aileen Starks

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

May 2022

Approved By

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Advisor: Professor Paul Boudreau

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Reader: Professor Patrick Curtis

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Reader: Professor Vitor Pomin

ACKNOWLEDGEMENTS

I would like to express my most sincere gratitude to my thesis advisor Dr. Paul Boudreau for his guidance and patience throughout these past two years of research and for giving me the chance to build my scientific knowledge and critical thinking abilities.

In addition, I wish to extend my special thanks to my committee readers, Dr. Patrick Curtis and Dr. Vitor Pomin, for their constructive feedback which helped to develop this Thesis.

Lastly, I would also like to show my appreciation for my fellow peers, Mohammed Ahmed, Tahir Ali, Alexis Wagner, Lana Taylor, David Uribe, and Braxton Crumpler. You all have been so helpful and encouraging throughout this research, and I am forever grateful.

ABSTRACT

VICTORIA STARKS: A Comparison of Heterotroph Isolation and Sequencing Methods From Various Cyanobacterial and Algal Microbiomes

(Under the direction of Paul Boudreau)

Cyanobacteria have provided a vast, new source of natural products to be utilized in drug development. Because of their non-axenic nature, cyanobacteria typically have an abundance of symbiotic heterotrophs living in association with them. These bacteria can play significant roles in the survival of its cyanobacterial host as well as provide the potential production of unique compounds. The possibility of unknown natural products is only increased by the flexible nature of these bacteria, as altering its environmental state can change the activity of biosynthetic pathways and even activate novel production. Our research team's intent is to isolate cyanobacterial strains from diverse sources and investigate for the production of unique compounds. Furthermore, the team aims to isolate individual heterotrophic symbionts from the cyanobacterial culture and determine their identity through sequencing. To date, the lab has collected 49 heterotrophic bacteria strains from local sources as well as 45 from various library strains. Furthermore, the lab has conducted many methods of heterotrophic isolation and sequencing preparation and has determined the effectiveness of each approach. Future work on identifying heterotrophic sources of unique compounds and understanding their potential uses will provide novel resources to be utilized in drug development. Overall, the goal of the lab is to identify and harness unique natural products to inspire production of new treatments to improve human health.

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INTRODUCTION

Natural products have been seen as a valuable source of unique compounds to be utilized for drug development (Newman & Cragg, 2020). Cyanobacteria are a unique clade of prokaryotes that are found in diverse environments all around the world (Moss, 2020). Cyanobacteria themselves play a unique role in many ecological processes and are a rich source of bioactive natural products (Gerwick & Moore, 2012). Perhaps the most interesting field of research in this area deals with the heterotrophic symbionts associated with the cyanobacteria, both free living and bacteria attached to the cyanobacterial host (R. A. Hughes et al., 2018). Throughout the early stages of research in the Boudreau Lab, our team focused its primary efforts on isolating an array of cyanobacterial strains, both collected from local sources and from culture libraries, and analyzing the metabolome of these organisms through Mass Spectrometry. The next step in this investigation was to determine the presence of heterotrophic bacteria in the cyanobacterial assemblage. These heterotrophs may be responsible for the production of unique compounds, either directly or by inducing their cyanobacterial host to produce new chemistry. So we set out to isolate heterotrophic bacteria from the cyanobacterial cultures, later identifying those bacteria through 16S gene sequencing. Our research stems from previous work analyzing the natural products and the heterotrophic microbiome associated with the cyanobacterial strain Moorea producens JHB (formerly Lyngbya producens JHB), which was collected from Hector's Bay, Jamaica in 1996 (Boudreau et al., 2015) (Cummings et al., 2016). This thesis focuses on the

actions taken to isolate cyanobacteria and their microbiome and analyzes the usefulness of various sequencing preparation methods employed to identify these organisms.

Unique Properties of Cyanobacteria

Cyanobacteria are an abundant group of bacteria found in many different types of environments (Moss, 2020). These bacteria have the ability to engage in photosynthesis and play a significant role in a multitude of ecological functions, such as serving key roles in both the carbon and nitrogen cycles, which fueled previous cyanobacterial research on tropical sponges (Schorn et al., 2019). For example, specialized cells in some filamentous cyanobacteria, heterocysts, contain the enzyme, nitrogenase, which allow them to fix dinitrogen (Hube et al., 2009). In addition, cyanobacteria have even played a monumental role in the diversity and evolution of most species on Earth through oxygenic photosynthesis, creating Earth's oxygen rich atmosphere, which keeps most species on earth alive (Leao et al., 2017) (Moss et al., 2018).

In addition, isolated cyanobacteria are typically xenic, meaning heterotrophic bacteria have been found living in association with the cyanobacteria. Multiple studies on cyanobacteria have led to the conclusion that the bacteria's polysaccharide and peptide containing envelops could provide the ideal environment for heterotrophic bacteria (Hube et al., 2009), but other symbiotic heterotrophs have been found as free-living bacteria within a cyanobacterial culture, unattached to filaments (R. Hughes et al., 2017). The role of these heterotrophic bacteria has been the topic of study for many years, as they can play significant roles in biosynthetic pathways through the production of signaling molecules or nutrient exchange (Cummings et al., 2016). Some of these products, such as chemical defenses and nutrients, can be crucial both for the survival of the heterotrophic bacteria and the cyanobacteria (Schorn et al., 2019). Associated heterotrophs benefit from the carbon and nitrogen fixating ability of cyanobacteria and may even play a role in these cycles, for example, studies have shown heterotrophic bacteria may lower the oxygen levels in the environment in order to keep the oxygen-sensitive nitrogenase functioning

at its highest rate (Hube et al., 2009). Because of this major interdependence, culturing cyanobacteria alone, or culturing its heterotrophic symbionts alone, can prove to be quite difficult as was experienced in our own lab.

Furthermore, the microbial composition surrounding a specific cyanobacterial strain can be influenced by environmental conditions, and these heterotrophic bacteria have been known to modulate gene expression of cyanobacteria, changing, and sometimes even ceasing, the activity of some biosynthetic pathways (R. Hughes et al., 2017). Not to mention, these associated heterotrophs could also be the primary source of interesting natural products, rather than the cyanobacteria itself. Therefore, it is important for this type of research that individual associate strains are identified. In studies by the Hughes group, the presence of heterotrophs was determined by using a scanning electron microscope which revealed many bacteria around and attached to the cyanobacteria filaments and then running metagenome analysis (R. Hughes et al., 2017). Metagenome analysis studies genetic material directly from a mixed source, which contain many species in this cyanobacterial research due to the cultures being xenic. Although more is known about cyanobacteria, at the origin of natural products research was much larger organisms, such as sponges and red algae that produced unique compounds such as the ones we hope to uncover. However, as research efforts continued, the focus shifted to smaller organisms, spurring research such as our own, aiming to better understand their microbiome and metabolic processes (Gerwick & Moore, 2012). Even more interesting, the sheer diversity of cyanobacteria add to the curiosity of their potential, because as of 2012, only 1% of bacteria inhabiting the oceans have been discovered (Gerwick & Moore, 2012) leaving countless other proposed heterotrophic symbionts unknown. Identifying the members of the heterotrophic microbiome

within cyanobacterial assemblages is the first step in understanding and applying the role these organisms play in the production of unique natural products.

Natural Products and their Potential Applications

Because cyanobacteria are generally unable to grow alone, it is important to identify the other bacteria present as well as the role they are playing in biosynthetic pathways, most importantly for drug discovery, through the production of secondary metabolites. Because of the variability in regulation of the genome, the production of these natural products can depend on the bacteria's environmental conditions, which opens the door to an abundance of unique metabolites from a single source (Boudreau et al., 2015). Natural products research is growing in importance given the rise in antibiotic resistance, rendering some of medicine's most powerful tools useless in the future (Schorn et al., 2019). In addition, perhaps the most enticing reason for studying these natural products is the exhibition of novel activity by many of these metabolites (Gerwick & Moore, 2012). For example, discovered cyanobacterial natural products, tolyporphins, identified from a cyanobacterial strain collected from Micronesia serves as an efflux pump inhibitor and can act on tumor cells, showing to be phototoxic to eukaryotic cells and demonstrating anticancer activity (R. Hughes et al., 2017). Inspired by this, other useful secondary metabolites have been identified with similar cytotoxic activity, such as the jamaicamides and hectochlorins produced by *M. producens* JHB (Boudreau et al., 2015). These natural products were also employed for their anticancer activity, and were shown to be effective against small lung cancers and carcinoma of the nasopharynx in humans (Boudreau et al., 2015). More notable, however, is the appreciation that the cyanobacterial genus *Moorea* has led to the identification of more than 190 compounds over the previous two decades, a richness few other

genera can match (Leao et al., 2017). Furthermore, many of these natural products have been discovered through analysis using high pressure liquid chromatography, absorption chromatography, and mass spectrometry (R. Hughes et al., 2017), which was also used by our team. Although only a small portion of the natural products bacteria can produce have been identified and harvested, there is significant evidence revealing that diverse marine environments may provide a pool of new chemical entities with the potential application of biotechnology (Cummings et al., 2016). Supporting this contention, 26 new drugs approved between 1981 and 2011 were based on natural products and used for a variety of treatments including, antitumor agents, treatment for Hepatitis C Virus, countering nausea from chemotherapy, and even a cardiovascular treatment, which emphasizes the importance of isolating novel natural products (Newman & Cragg, 2020).

The Role of the Microbiome

Although research has shown the potential for an abundance of cyanobacterial derived natural products, very little is actually known about the ecological role of these products, or why some biosynthetic pathways expressed and some are silent (Leao et al., 2017). It is assumed the microbiome plays a role in the activation of these natural product biosynthetic gene clusters or their inhibition. *Moorea*, as mentioned previously, have a large capacity for producing natural products originating from genomes that bear a large number of biosynthetic gene clusters. (Leao et al., 2017) When looking specifically at *Moorea producens* PAL, 91% (40 out of 44) of gene clusters only match cryptic clusters, which are gene clusters only found in one strain of bacteria with no matches in the NCBI database (Leao et al., 2017). Furthermore, when compared to other strains of *Moorea*, researchers found nearly 16% of the gene clusters were orphan clusters, which

mean they are only found in one specific strain from this genus. The uniqueness of these clusters provide evidence suggesting they likely code for new natural products, but it is still unknown what natural products many of these *Moorea* gene clusters produce (Leao et al., 2017). Understanding the role and activation of these genes by environmental conditions or interactions with other bacteria will play a key role in helping to explore this novel chemical space.

Further studies on *M. producens* JHB showed multiple strains living within the culture years after collection from Hector's Bay, Jamaica (Cummings et al., 2016). By seeking the genome for the cyanobacteria, data from the entire microbiome community was collected from the xenic culture, Cummings and coworkers found a resident microbe based on GC content analysis. The GC content from the cyanobacteria alone was 43.5% while the other contig's GC content was 66.8%, they dubbed this microbiome member Mor1 (Cummings et al., 2016). Moreover, Cummings and Barbé's comparison of *M. producens* and Mor1 revealed unique genes found in the heterotrophic bacterium and not in the cyanobacteria, demonstrating the need for understanding the microbiome composition (Cummings et al., 2016). Without discovering and isolating the diversity of heterotrophs present within the individual microbiome of a cyanobacterium, it remains a challenge to know the true source of these natural products and whether or not other heterotrophic bacteria are needed to turn on specific biosynthetic genes in the cyanobacterial host. Our research aims to better understand the microbiomes of our cyanobacteria cultures by discovering the key players in the microbiome by isolating and identifying each cyanobacteria's heterotrophic symbionts.

RESULTS AND DISCUSSION

Algal Strain Isolations

Culture Preparation Methods

Through trial and error, the most efficient methods for the isolation and identification of strains were discovered . The degree of dilution from the environmentally derived samples varied throughout the course of study. Although growth was observed on most concentration levels, the speed and amount of growth differed with concentration, and lower dilutions showed the most efficient growth. For the original environmentally derived strains where dilutions of 10,000 x, 100 x, and 1 x were tried, the highest yield of algal colonies came from the undiluted samples, which was the case for BL-A-01. Moving forward, only more concentrated samples were used to grow cultures, so for isolations from fresh water sources in northern Mississippi, only the raw sample and a 10 x concentration were tested, both yielding colonies with water samples from Enid Lake, Grenada Lake, and the Tennessee Tombigee Waterway.

In addition to starting concentration, media also influenced the growth of cultures. Both freshwater J medium and freshwater BG-11 medium were used to enrich algae, but BG-11 was proven to be more useful. The majority of isolates in this research were prepared from

samples grown on BG-11 media. The only exception was BL-A-01, as it efficiently grew on both types of minimal media.

Observations of Algal Cultures

The period of time needed for strains to grow varied greatly among cultures. Growth time on plated media typically took about a month for colonies to become visible, as cyanobacteria are typically very slow growing. Strains typically grew quicker when transferred to liquid media than they did on plated media. In addition, morphologies varied in size and color. Some morphologies were lighter green in color while others were a darker green. These morphologies varied even more when placed in liquid media for long-term cultivation, as some showed a matted morphology while others grew suspended in the liquid medium. Other factors, such as light intensity and media concentrations were held constant, leading us to be able to attribute varying morphologies to different strains rather than environmental influences.

Cyanobacterial 16S PCR Optimization

Determination of Effective Primer Pairs

A central goal during this stage of research was finding a forward and reverse primer pair that was able to amplify the 16S gene specifically from a cyanobacterium while avoiding amplification of heterotrophic bacteria in the assemblage and also avoiding off target amplicons not at the expected length. Through primer trials, it was found, as expected, that universal primers (TS05 and TS06) were unsuccessful in selecting specifically for cyanobacterial DNA, as

only sequences from heterotrophic bacteria were detected. In the trial of TS02 with TS07, a cyanobacterial specific forward primer and universal reverse primer, heterotrophic bacterial 16S DNA was amplified. This result was observed even though TS02 was a cyanobacteria-specific primer designed off the work of Nübel and coworkers (Nübel et al., 1997). The universal primer, TS07 (utilized in the work by Moss and colleagues), and a single nucleotide wobble off of TS02 allowed for amplification of heterotrophic bacteria DNA, which was not beneficial for this study (Moss et al., 2018). This problem was observed on PCR amplification of the 16S gene from BL-A-01, which yielded sequences from a heterotrophic bacterium (by BLAST was closest to *Tetrasphaera* sp). Successful amplification of the cyanobacterial 16S gene alone was acquired when using the TS02 forward primer and the TS03+TS04 reverse primers, as predicted due to their cyanobacteria specific sequences. Going forward, all other cyanobacterial identification efforts were conducted using these primers and the conditions set out in the methods section.

Algal Sequencing Data

Although gene amplification was evident, some of the sequencing data from our cultures resulted in strikingly low pairwise alignment with public bacterial 16S sequences, leading us to question the identity of the of these supposed cyanobacteria. For some strains, such as BL-A-01, the pairwise sequence identity was around 85%, but with so many sequenced strains of cyanobacteria we would expect species level matches between 98-100% pairwise identity. These results were due to cultures actually containing eukaryotic algae strains rather than cyanobacteria. This was shown when sequencing data was aligned with sequences from chloroplast DNA sequences, resulting in an increased pairwise function of around 94%, much higher than shown in comparison to cyanobacterial 16S gene sequences. This is due to the endosymbiotic evolutionary history of cyanobacteria, as the origin of chloroplast evolved from

an engulfed cyanobacterium (Nübel et al., 1997). Because of this, cyanobacteria primers are still able to bind with DNA from the chloroplasts with high affinity, contributing to confounding results.

Heterotrophic Isolation from Cyanobacterial and Algal Strains

Media Comparisons for Heterotrophic Isolation

In total, 49 heterotrophic bacteria frozen stocks were prepared from isolates of environmentally derived algal strains and 45 frozen stocks were prepared from isolates of cyanobacterial library strains. These heterotrophs varied greatly in their colony morphology and coloration, ranging from clear to pink to brown. Many types of media were used during heterotrophic isolation varying in their carbon and nitrogen sources to stimulate multiple environmental conditions which could influence the growth of individual microbiome components. Changing the nutrients available across these media was done to yield the highest number of heterotrophic isolates as possible as nutrient demands can vary across species (Nguyen, 2018). From **Figure 1**, it is observed that LB and TB media were able to grow the highest number of strains with 9, out of the 12 total, different heterotroph species showing growth on these media. This panel of media also revealed some isolates, *Flavobacterium* and *Pseudomonas,* were able to grow on a wide variety of media, proving to be the most flexible strains in this study in terms of media choice. Going along with this, *Flavobacterium* demonstrates the importance of utilizing a wide panel of media. Two separate species of *Flavobacterium* were cultured from environmentally derived samples, and although they were part of the same genus, they grew on different types of media, both nutrient rich ones like TB as

well as those with less nutrients and varied components such as DMD, R2A, and NRRL13. Comparing these media types shows the wide range of conditions suitable for the strain, and demonstrate that although the carbon and nitrogen sources vary, which were the main means of comparison, other seemingly minor components may be the reason even strains that appear more flexible are unable to grow on some media but grow easily on others. Another important conclusion from this study was the evidence for ISP-3 and ISP-4 culturing comparatively rare bacteria. *Chryseobacterium, Rhodococcus*, and *Bacillus* were the only strains shown to grow on these media types, and therefore, using these media types could be beneficial in the future for selectively culturing more members of the microbiome.

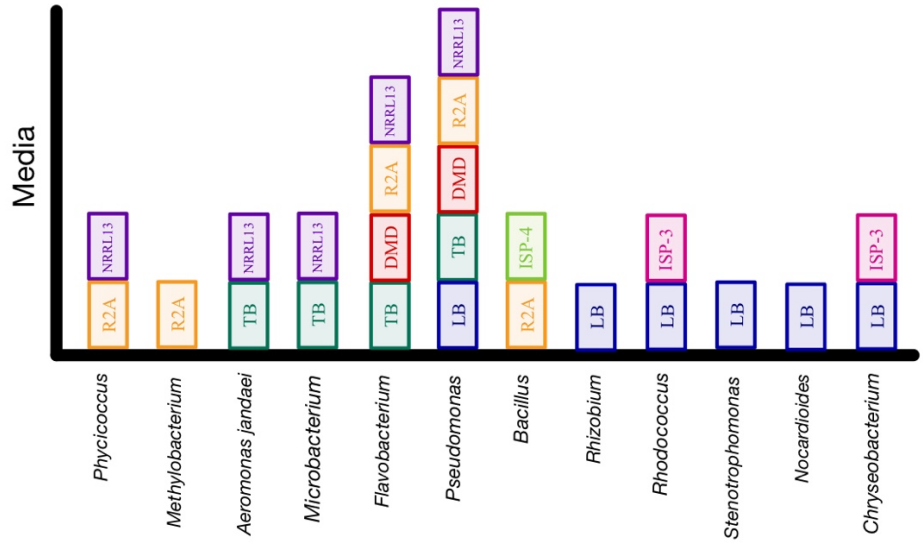

Figure 1 shows isolated and identified heterotrophic bacteria strains along with the types of media it was cultured from. It is important to note, LB and TB consist of the same carbon (tryptone) and nitrogen (yeast extract) sources.

Heterotrophic Isolates from Algal and Cyanobacterial Strains

Comparing media-associated heterotrophic growth was not the only domain of study, it was also important to analyze the link between the given algal or cyanobacteria strain and its

resident heterotrophs, which can be seen in **Table 1**. One important trend seen in this table is the commonality of *Flavobacterium* spp. being found within BL-A-02, BL-A-04, and BL-A-09. Because all three strains were cultured from samples from Grenada lake, this commonality could be due to the shared environment from which they were collected. Because heterotrophic bacteria are known to live on and around cyanobacterial filaments, it is reasonable to assume this similarity is due to *Flavobacterium* being present in the environment and attaching to these filaments.

Despite collecting isolates from multiple cyanobacterial and algal strains, culturing isolates from some proved unsuccessful, such as isolating heterotrophs from the culture library strain ATCC 29413. Although isolates grew on plated media, when transferred to liquid media to prepare for sequencing and frozen stocks, growth was not observed after giving 2-3 days to grow, which was the standard time period for other heterotrophs to grow. The only growth seen from this strain was on DMD media, so in order to prepare samples for sequencing of heterotrophs from the ATCC 29413 community, our lab will need to expand our media panel to include new media similar to DMD as well as trying other more diverse media. This could include using other medias containing pyruvic acid or L-glutamine, along with modification to DMD in which one of these is held constant while another component is substituted.

	Phycicoccus	Methylobacterium	Aeromonas jandaei	Microbacterium	Flavobacterium	Pseudomonas	Bacillus	Rhizobium	Rhodococcus	Stenotrophomonas	Nocardioides	Chryseobacterium
BL-A-01	✓	✓										
BL-A-02			✓	✓	✓							
BL-A-03						✓						
BL-A-04					✓							
BL-A-09					✓							
BL-A-16						✓						
UTEX LB3037							✓	✓				
UTEX B1270									✓	✓		
UTEX B629											✓	
UTEX B1567							✓					✓

Table 1 shows heterotrophic bacteria strains isolated from each specified algal or cyanobacteria strains. It includes all environmentally derived and culture library strains.

Heterotrophic DNA Isolation and Sequencing

Comparison of DNA Preparation Methods

Over the course of study, a constant goal was discovering the most efficient means of DNA preparation for heterotrophic constitutes isolated from the cyanobacterial or algal microbiome. Using cells submitted as glycerol stocks with a commercial vendor (GeneWiz) led to many poor Sanger sequencing results. Hypothesizing that the concentration of DNA within the sample was less than desired for the commercial vendor's sequencing process, our samples prepared for sequencing were prepared using three times the original concentration of cells. Despite having an increased amount of successful sequencing results, many continued to come back with low quality reads or yielded very short sequences, making it difficult to confidently identify these strains. This could be due to a variety of factors, such as aged or dying cells in the cultures, stocks mistakenly containing more than one heterotroph species, or inaccessibility of DNA during commercial vendor's cell lysis processing step.

Combatting the last issue was the goal of utilizing the Omega-Biotek EZ DNA Isolation Kit in our lab, as it would produce high purity DNA samples we could submit directly. On the first batch of sequencing data acquired from DNA purified in this manner, over half of the samples were returned with high quality DNA sequences matching strains well to sequencing in public databases. This lysis set allowed us to submit genomic DNA of known quality and quantity, as the cell was lysed and DNA was purified before sending to the commercial sequencing company, leading to more accurate data from their Sanger sequencing process. Although the amount of sequence reads using this was higher, still some inconclusive results were observed. A heterotrophic strain (BL-A-01-R2A-H3) isolated from BL-A-01, an algae isolated from the root of an orchid plant, had sequencing data aligning with a strain of

Methylobacterium. However, in our original analysis the returned sequence was much shorter than the rest, leading us to question the validity of the read. Closer investigation showed a single base ambiguity in the middle of the read. Including this ambiguity produced a longer sequence, and when repeating the BLAST search for this sequence, still revealed a pairwise identity of 99.7% with the *Methylobacteria* species and had a much longer coverage. In the future, it should be a goal to go back and try to re-identify this strain to see if this conclusion is replicable.

In spite of using the EZ Bacterial DNA Kit, some preparation methods yielded low concentrations when measure on the QuBit, which utilizes a fluorescent dye attaching to purified dsDNA samples to measure sample DNA concentrations. Some of these concentrations were far below the recommended concentration of 50 ng/μL from GeneWiz, with concentrations as low as 0.183 ng/μL, which were far below the amount needed for successful sequencing. To combat this issue, later attempts adopted the manufacturer's optional bead beating step, leading to overwhelming increases in DNA concentration prepared by this method, 4050 to 8210 ng/μL, which could contribute to better sequencing outcomes. This method led to much higher confidence in sequence data by promoting better binding of DNA molecules to the beads during isolation, making it a much purer and more concentrated sample.

Isolate Identification from Sequencing Data

Overall, the procedure utilizing the DNA isolation and purification led to much higher quality reads and more conclusive sequencing. One major strength of this sequencing preparation method was the ability to distinguish not only various heterotrophic bacteria strains, but also strains within the same species. This is best seen in heterotrophs from the algal strain BL-A-02.

As demonstrated on **Table 2**, which shows the most closely related heterotroph species determined through sequencing, within this singular microbiome, two different strains of *Flavobacterium* were isolated. Both strains were able to be isolated on DMD media, but *Flavobacterium* sp. Candidate 1 was also cultured on NRRL-13, whereas *Flavobacterium* sp. Candidate 2 was not observed to grow on this media. This result alone supports the advantage of the specificity provided through this isolation and sequencing, as this is necessary to fully understand the makeup of the algal microbiome and eventually utilize these constituents to identify and harvest vast natural products.

After sequencing was completed by GeneWiz, the long sequences collected were put into a BLAST search to compare sequence similarities to all strain documented in the database as well as compared to sequences acquired from other strains. The latter was important in recognizing whether we were collecting strains that were actually different or in the same genus. This was best seen in sequencing results of BL-A-16. All six isolated samples returned sequencing aligning with those from the *Pseudomonas* genus with a 100% pairwise identity. Pseudomonads are a very common bacteria, but there are many different strains. When compared to the *Pseudomonas* species isolated from BL-A-03, the two were very similar, but these two *Pseudomona*s spp. only shared a 96.3% pairwise identity, showing that closely related bacteria could be isolated from different algal hosts **(see Table 2)**.

Lab Code	Hit Species	Hit Name	% Pairwise Identity
BL-A-01-R2A-H3	*Methylobacterium sp.*	NR_041443	99.7
BL-A-02-NRRL13-H1	*Aeromonas jandaei sp.*	NR_119040	100
BL-A-02-TB-H1	*Aeromonas jandaei sp.*	NR_119040	100
BL-A-02-TB-H1B	*Aeromonas jandaei sp.*	NR_119041	100
BL-A-02-DMD-H3	*Flavobacterium tistrianum* GB56.1 (Candidate 1)	NR_109024	99.1
BL-A-02-DMD-H2	*Flavobacterium anhuiense* D3 (Candidate 2)	NR_044388	99.9
BL-A-02-TB-H2	*Microbacterium paraoxydans* CF36	NR_025548	99.8
BL-A-03-DMD-H1	*Pseudomonas canadensis*	NR_156852	99.9
BL-A-03-DMD-H2	*Pseudomonas canadensis*	NR_156853	99.9
BL-A-04-DMD-H1B	*Flavobacterium tistrianum* GB56.1	NR_109024	99.1
BL-A-04-DMD-H2	*Flavobacterium tistrianum* GB56.1	NR_109024	99.1
BL-A-04-DMD-H2B	*Flavobacterium tistrianum* GB56.1	NR_109024	99.1
BL-A-04-DMD-H2C	*Flavobacterium tistrianum* GB56.1	NR_109024	99.1
BL-A-09-TB-H1	*Flavobacterium anhuiense*	NR_044388	99.9
BL-A-09-TB-H2	*Flavobacterium anhuiense*	NR_044388	99.9
BL-A-09-DMD-H1L	*Flavobacterium anhuiense*	NR_044388	99.9
BL-A-09-DMD-H1L1	*Flavobacterium anhuiense*	NR_044388	99.9
BL-A-16-LB-1	*Pseudomonas punonesis*	NR_109583	99.6
BL-A-16-LB-2	*Pseudomonas punonesis*	NR_109583	99.6
BL-A-16-DMD-1	*Pseudomonas punonesis*	NR_109583	99.6
BL-A-16-DMD-2	*Pseudomonas punonesis*	NR_109583	99.6
BL-A-16-R2A-1	*Pseudomonas punonesis*	NR_109583	99.6
BL-A-16-R2A-2	*Pseudomonas punonesis*	NR_109583	99.6
LB3037-ISP3-2B	*Bacillus pacificus*	NR_157733	100
LB3037-ISP4-3	*Bacillus pacificus*	NR_157733	100
LB3037-ISP4-3	*Bacillus sp.*	NR_157733	100
LB3037-R2A-2	*Bacillus sp.*	NR_157735	100
UTEX-LB3037-4	*Rhizobium sp.*	NR_116445	99
B1270-H6	*Rodococcus qingshegii*	NR_115708	100
B1270-H8	*Stenotrophomonas pavanii*	NR_118008	99.9
B629-H1	*Nocardioides plantarum*	NR_119354	98.4
B1567-H3	*Bacillus oceanisediminis*	NR_118440	100
B1567-H6	*Chryseobacterium aquaticum*	NR_042642	100

Table 2 shows BLAST his examples for some lab strains sent to GeneWiz sequencing along with the closest matched sequence and its pairwise identity.

FUTURE DIRECTIONS

Continuation of Heterotroph Isolations

Based on the evidence presented by **Figure 1** in the results section, the type of media used impacted ability for different types of heterotrophs to grow, and throughout the collection of heterotrophs from cyanobacterial cultures, many picked colonies were later found to be the same strain. Hypothesizing these findings are due to lack of diversity in media composition, future work should be conducted utilizing more specialized types of media. For example, two media types used, LB and TB, were composed of the same nitrogen and carbon sources, so moving forward, the goal is to stop using TB and replace it with another media with the potential to grow previously uncultured heterotrophic constituents of the cyanobacterial microbiome. One proposed method of media incorporation follows the direction of Nyugen et al., incorporating other components, such as diverse amino acids and fatty acids to attempt to grow bacteria with specialized media demands (Nguyen, 2018). This group proposed a method of extracting soil nutrients from the environment in which the bacteria was collected, which is meant to mimic environmental conditions, encouraging the growth of more diverse heterotroph populations. This could potentially eliminate a key shortcoming of this work, which is the inability to grow the entire array of cyanobacterial microbiome strains within laboratory conditions. Specifically, this could be beneficial for culturing heterotrophs from cyanobacterial strains such as BL-A-16. Although growth and isolation of heterotrophs was possible, all isolated strains were Pseudomonads rather than different strains. Incorporating soil nutrients, amino acids, or fatty

acids into the media could prove invaluable in collecting previously uncultured strains that need nutrients that may have been previously unavailable in our media panel.

In addition, in comparison to minimal media used to culture cyanobacteria, heterotroph media is comprised of much more nutrients, as the lack of photosynthetic ability makes it necessary to provide a carbon source. However, an important component of cyanobacterial media, such as freshwater BG-11 and FW-J, are trace metals. Trace metals provide important nutrients needed to perform cyanobacteria functions such as photosynthesis and nitrogen fixation. Likewise, heterotrophs present in the microbial community may also be using these nutrients for their various biosynthetic pathways. Despite this, no currently used heterotroph media contains the same specific trace metals as our cyanobacterial media, which could be contributing to difficulty culturing diverse symbionts. Going forward, adding trace metals such as the BG-11 trace metals mix should be incorporated in experiments to potentially isolate and identify new heterotrophs. Furthermore, another difficulty faced throughout this research was combatting fungal contaminants. For many heterotroph strains, fungal contaminants appeared on the plated media. When this occurred, the plates had to be discarded, which could have also led to the loss of newly cultured heterotrophic bacterial strains. Many times, it was impossible to retrace the steps necessary to re-isolate the strain on a new plate, so for most, efforts ceased if this occurred. It was also unknown if these fungi were acquired from the lab or were previously living with the cyanobacteria with insufficient nutrients to grow until transferred onto nutrient-rich media. Adding an antifungal component to media, such as nystatin, could help combat this issue and should be incorporated in the future to prevent further loss of what could be newly cultured heterotrophs.

Extended Sequencing Efforts

A major challenge facing early stages of this research was ensuring amplification of the 16S gene of the cyanobacterial cultures. This involved trying a variety of forward and reverse primers repeatedly to find a combination that was effective. Bands at the correct length were observed while using each primer pair, but exclusive amplification of the cyanobacterial, as opposed to heterotrophic strains', 16S gene was only seen while using both cyanobacterial specific forward and reverse primers. Because of these findings, in upcoming experiments, researchers should use only the cyanobacterial specific forward and reverse primers, as they were the only combination leading to consistently accurate amplification, whereas universal primers (or a combination of the two) showed no amplification or amplification of heterotrophs living within the microbiomes.

On the other hand, multiple sequencing preparation methods were experimented with while striving to obtain clean reads of heterotroph 16S sequences. Early on, much of the sequencing efforts were tried using direct cell pellets from heterotroph cultures, but much of the data returned when preparing samples in this manner were returned with poor reads. This could be due to a variety of factors such as cultures accidentally containing more than one heterotroph or the heterotrophs being too diluted and not having a high enough concentration of genetic material. To counter with this issue, other preparation methods were tried, and the most successful was the one using the EZ Bacterial DNA kit for cell lysis and purifying DNA samples. Proceeding with this research, all other sequencing trials should be conducted using this method, utilizing the optional beading step to increase yield, as it was the most efficient approach of those tried and also led to more reliable identifications when analyzing BLAST hits.

In addition, it would be constructive to revisit the DNA preparations for samples showing lower quality results during sequencing.

LCMS Analysis of Cyanobacterial Cultures

To date, this research has provided a better understanding of the microbial community associated with various cyanobacteria strains. To build upon this work, the next direction should be to investigate the chemistry associated with this now better understood microbiome. At the beginning of this work, this was the original intent, but the results observed were insignificant, so it was crucial to step back and better understand what heterotrophs lived within the cyanobacterial microbiome. This work is best conducted through the use of liquid chromatography-mass spectrometry (LCMS). This technique separates compounds by their mass and can fragment the molecular ions to provide insight into their chemical structure, where the fragment spectra can then be compared across multiple samples using the molecular networking tool (Wang et al., 2016). This would be advantageous to this line of study due to the ability to compare compounds across samples in order to identify masses of unique compounds, which appear as a separate cluster when all are mapped together. After identifying unique compounds, knowing the heterotrophs present in each could help identify the source, or drivers, of unique natural products. On the other hand, for more common compounds, it would be possible to then identify if they derive from a similar bacteria, as the heterotroph components of the microbiome are well on their way to being documented. Lastly, this would be advantageous, because it would help narrow the scope of heterotroph isolations, instead focusing only on those deriving from cultures showing unique natural products.

Along with LCMS analysis, altering the environments of the cyanobacteria could lead to changes in the microbial community and therefore changing the chemistry occurring within it, which has begun to be explored within the Boudreau Lab. Ways the environment could potentially be altered is through changes in salinity, antibiotic stressors, and halogen ion concentrations. After changing these, LCMS should be re-run to compare compounds and determine if new compounds are being produced, as manipulating the external environment could alter gene expressions, turning some biosynthetic pathways off or on within the cyanobacteria and associated heterotrophs, which could potentially could lead to the production of new natural products.

METHODS

Isolating Algal Strains

To begin our research, samples were first collected from various sources, such as the original orchid and carnivorous plant sources. 10 mL of sterile MilliQ water was passed through the roots of the plant, and then the water was collected from the bottom of the plant using a pipette. After the water was collected, three different samples were prepared: an undiluted, a 100x diluted, and a 10,000x diluted. The 100x diluted was prepared by adding 10 μL of the water passed through the plant roots into 990 μL of sterile PBS. The 10,000x dilution was prepared by adding 10 μL of the 100x dilution into 990 μL of sterile PBS. 25 μL of each sample was then streaked out onto solid fresh water J or BG-11 media. The plates were placed under a 1550 lux grow light and allowed to grow for approximately a week. After this time, the colonies were restruck onto a new plate containing the original type of medial using the loop method. The loop method involves picking an individual colony using a sterile loop and then spreading it across agar plates under sterile conditions. If the second plate is determined to only contain a single morphology, another colony was picked using a sterile loop and placed into a sterile flask containing 50 mL of liquid media, either J or BG-11 according to the plate. Again, the liquid cultures were placed under the 1550 lux grow light. These liquid cultures were regularly passaged into fresh media to maintain a healthy culture to be used for later experiments. Later strains from other environmentally derived strains were collected from Sardis, Enid, and Grenada Lakes. The cultures were prepared and maintained using a similar procedure as those from the

plants, but the method was slightly changes in the dilutions. Two different concentrations were streaked onto fresh water J or BG-11 media plates. One was 50 μL of undiluted sample, while the other was concentrated x10 by centrifugation. Again, cultures were grown under grow light and maintained by passaging regularly.

Lastly, strains were purchased from culture libraries and grown according to the instructions provided from the library, and were subjected to the same course of growth as previously noted strains. Cyanobacterial strains selected for purchase were non-axenic strains, meaning they had not been isolated from other heterotrophs, as the goal of this study was to identify unique constituents of the microbial community. The strains obtained from the American Type Culture Collection (ATCC) and University of Texas (UTEX) are listed in **Table 3.**

Lab Code of Strain	**Cyanobacteria Strain**
ATCC 29413	*Trichormus variabilis*
UTEX LB3037	*Microcystis aeruginosa*
UTEX B1270	*Oscillatoria prolifera*
UTEX B629	*Anabaena cylindrica*
UTEX B1567	*Ocillatoria brevis*

Table 3 lists lab code of each library strain and the species of cyanobacteria.

Optimization of Cyanobacterial 16S PCR

In a PCR tube, the following were mixed: 0.5 μL forward primer, 0.5 μL reverse primer, 0.5 μL of liquid culture, 11.0 μL MilliQ water, and 12.5 μL of 2x Master Mix Q5 polymerase (New England Biolabs). Each PCR sample was run on the thermocycler with the following cycle: 98° C for 30.0 seconds, 98° C for 5.0 seconds, 61° C for 10.0 seconds, 72° C for 40.0 seconds, 72° C for 2.0 minutes, and 4.0° C for the remainder of time. Steps 2 through 4 were repeated 30 times. 1x TAE buffer was placed into the gel rig, and the samples were run on a 1% agarose TAE gel for 25 minutes at 100 volts. Additional primers, voltage, and runtime were modified until the bands at the expected length were amplified. The samples were placed in the thermocycler on a gradient of the optimal annealing temperatures, which are listed below. It is important to note that TS03 and TS04 were combined into a single, reverse primer (Nübel et al., 1997). Forward Primers TS01 and TS02, and reverse primers TS03 and TS04 were cyanobacterial specific primers designed by Nübel et al. TS05 and TS06 were general bacterial 16S sequences from (Herlemann et al., 2011). The designation of 16S primers given in the literature are also noted below in **Table 4**. Each primer was used because of evidence of success within other studies (Nübel et al., 1997) (Herlemann et al., 2011). Finding primers specific enough to only show cyanobacteria genes, while also utilizing cyanobacteria specific primers that still allowed for a wide array of cyanobacteria strains could prove difficult, but in the literature, these were shown to produce 16S bands of the expected lengths. Lastly, it should be pointed out that although TS05 and TS06 were investigated as possible primers and used when determined an efficient PCR procedure, there is no further data collected from sequencing using these primers.

Primers	Primer Sequence	Annealing Temperatures
TS01: Full CYA106F	CGCCCGCCGCGCCCCGCGCCGG TCCCGCCGCCCCCGCCCGCGGA CGGGTGAGTAACGCGTGA	TS01 & TS06: 44°C
TS02: Short CYA106F	CGGACGGGTGAGTAACGCGTGA	TS01 & TS03+TS04: 52°C
TS03: CYA781R(a)	GACTACTGGGGTATCTAATCCC ATT	TS02 & TS03+TS04: 52°C
TS04: CYA781R(b)	GACTACAGGGGTATCTAATCCC TTT	TS02 & TS06: 44°C
TS05: Bakt 16S 341F	CCTACGGGNGGCWGCAG	TS05 & TS03+TS04: 52°C
TS06: Bakt 16S 805R	GACTACHVGGGTATCTAATCC	TS05 & TS06: 44°C
TS07: MM 16S 1509R	GGTTACCTTGTTACGACTT	TS02 & TS07: 52°C

Table 4 lists primers and sequences as well as the annealing temperature used for each forward and reverse primer combination

Heterotrophic Isolation from Cyanobacterial and Algal Samples

Under sterile conditions, a sample was collected from the algal liquid culture using a sterile loop and then was streaked onto a plate of the media in **Table 5**. It is important to note DMD media was designed in the Boudreau Lab.

Each plate was allowed to grow until individual colonies were visible. As different morphologies were noted on the plate, distinct colonies were picked onto another agar plate of the same medium using a sterile loop. The plates, again, were allowed to grow and were checked

to ensure that only a single morphology was growing on the new plate. If only a single morphology was present, a sterile loop was used to pick a single colony into a tube containing 2 mL of liquid medium. The liquid media used was the same as the solid media, and in addition, LB media was also tried in many cases. Tubes containing liquid cultures were placed on a shaker and allowed to grow for up to one week. A frozen stock was prepared to keep in the lab by adding 500 μL of liquid heterotroph culture to 500 μL of 50% glycerol stock in a cryogenic tube. The stocks were vortexed and then stored at -70 °C in the freezer.

Media	**Carbon Source**	**Nitrogen Source**
LB	Tryptone	Yeast Extract
TB	Tryptone	Yeast Extract
R2A	Glucose Soluble Starch	Proteose Peptone Casamino Acid
DMD	Pyruvic Acid	L-glutamine
NRRL-13	Mannitol	Yeast Extract
ISP-3	White Oats	White Oats
ISP-4	Soluble Starch	Ammonium Sulfate

Table 5 show the media panel used throughout this investigation as well as the specified Carbon and Nitrogen sources for each media.

Heterotroph DNA Isolation and Sequencing

After the heterotrophic bacteria were isolated from the cyanobacteria and grown in liquid media, initially a sample was prepared for sequencing using the same procedure for frozen stocks as

noted above. However, utilizing this method, many 16S sequencing attempts led to bad data with the commercial vendor. Because of this, a new method was tried using three times the concentration of cells, which yielded better sequencing results in some cases. For this preparation method, 1.5 mL of liquid culture was placed into a microcentrifuge and spun at 21xg at 13.0°C for 3 minutes. After, 1 mL of media was removed from the tip of the tube without disturbing the pellet. 500 μL of 50% glycerol was then added to the centrifuge containing the remaining media and cell pellet. The tube was vortexed and stored at -70 °C in the freezer until it was sent for sequencing. In cases where the sequencing data was still bad with a larger scale of cells sent to the commercial vendor, genomic DNA was isolated directly. Genomic DNA samples were prepared using the EZ Bacterial DNA Isolation Kit following the noted manufacturer's instructions. The concentration of DNA was then assessed using the QuBit. If the concentration was above 50 ng/ μL, the sample was sent for sequencing. If concentration of DNA was too low, genomic DNA preparation was redone altering the time of cell lysis or beating the cells with glass beads per the manufacturer's optional step.

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APPENDIX

A. Cyanobacterial Media

Fresh Water BG-11 Media

(Following protocol from the UTEX Culture Collection: https://utex.org/products/bg-11-medium?variant=30991786868826

Note the modification to mix stocks 5 and 6 together.)

Components:

$NaNO_3$ Stock (30 g / 200 mL)	10 mL/L
K_2HPO_4 Stock (0.8 g / 200 mL)	10 mL/L
$MgSO_47H_2O$ (1.5 g / 200 mL)	10 mL/L
$CaCl_22H_2O$ (0.72 g / 200 mL)	10 mL/L
Citric Acid Monohydrate (0.12 g / 200 mL) **AND** Ferric Ammonium Citrate (0.12 g / 200 mL)	10 mL/L
$Na_2EDTA2H_2O$ (0.02 g /200 mL)	10 mL/L
Na_2CO_3 (0.40 g / 200 mL)	10 mL/L
BG-11 Trace Metals Solution	1 mL/L
Sodium thiosulfate pentahydrate (49.6 g / 200 mL) [ONLY FOR AGAR PLATES]	1 mL/L

Trace Metals Solution:

H_2BO_3 [Boric acid]	2.86 g/L
$MnCl_2$ 4H2O [Manganese (III) chloride, tetrahydrate]	1.81 g/L
$ZnSO_4$ $7H_2O$ [Zinc sulfate, heptahydrate]	0.22 g/L
Na_2MoO_4 $2H_2O$ [Sodium molybdate, dihydrate]	0.39 g/L
$CuSO_4$ x $5H_2O$ [Copper (II) sulfate, pentahydrate]	0.079 g/L
$Co(NO_3)_2$ x $6H_2O$ [Cobalt (II) nitrate, hexahydrate]	0.0494 g/L

Protocol:

1. Add components in order to ~900 mL of MiliQ while stirring
2. If making agar plate, add 15 g of agar
3. Dilute to 1 L and autoclave

Fresh Water J Media

(Following protocol from the UTEX Culture Collection: https://utex.org/products/j-medium?variant=30991762653274)

Components:

Macro Component 1 for J Medium	10 mL/L
Macro Component 2 for J Medium	10 mL/L
$CaCl_2$ x $2H_2O$	1 mL/L
G9 Trace Metals for J Medium	1 mL/L

Macro Component 1:

Add the following in order to ~900 mL of MiliQ while stirring, adjust the pH to 8.5-8.8, and bring total volume to 1 L with MiliQ. Store in refrigerator.

$NaNO_3$	67 g/L
Na_2HPO_4	0.67 g/L
K_2HPO_4	3.8 g/L
Tricine	25 g/L

Macro Component 2:

Add the following in order to ~850 mL of MiliQ while stirring, adjust the pH to 7.5, and bring the total volume to 1 L with MiliQ. Store in refrigerator.

Na_2EDTA x $2H_2O$	4.4 g/L
$MgSO_4$ x $7H_2O$	10 g/L
$MgCl_2$ x $6H_2O$	5 g/L
$FeCl_3$ x $6H_2O$	0.5 g/L

G9 Trace Metals:

Add the following in order to ~900 mL of MiliQ while stirring and bring the total volume to 1 L with MiliQ. Store in refrigerator.

H_3BO_3	3.25 g/L
$MnSO_4$ x H_2O	1.5 g/L

$ZnSO_4$ x $7H_2O$	0.3 g/L
$(NH_4)_6Mo_7O_{24}$ x $4H_2O$	0.08 g/L
$CuSO_4$ x $5H_2O$	0.05 g/L
$Co(NO_3)_2$ x $6H_2O$	0.15 g/L
KI	0.01 g/L

Protocol:

1. Add components in order to ~950 mL of MiliQ while stirring
2. Bring total volume to 1 L with MiliQ (add 12.5 g of agar if making plates)
3. Autoclave and store in refrigerator

B. Heterotroph Media

Premixed LB Broth (Lennox) (premixed from Sigma-Aldrich)

Components:

NaCl	5 g/L
Tryptone	10 g/L
Yeast Extract	5 g/L

TB Media

(Following the Cold Spring Harbor Protocol:

http://cshprotocols.cshlp.org/content/2006/1/pdb.rec8620)

Components:

Yeast extract	24.0 g
Glycerol	4.0 g
Tryptone	12.0 g
Agar	15.0 g

Protocol:

1. Prepare Salt Solution of 0.17 M KH_2PO_4, 0.72 M K_2HPO_4
    a. Dissolve 2.31 g KH_2PO_4 and 12.54 g K_2HPO_4 into 90 mL MiliQ
    b. After salt has dissolved, dilute to a final volume of 100 mL
    c. Autoclave
2. Add media components to 900 mL of MiliQ
3. Autoclave and cool

R2A Media (DSMZ Medium 830)

(Following the protocol for DSMZ Medium 830:

https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium830.pdf)

Components:

Yeast Extract	0.50 g
Proteose Peptone	0.50 g
Casamino acids	0.50 g
Glucose	0.50 g
Soluble starch	0.50 g
Sodium pyruvate	0.30 g
K_2HPO_4	0.30 g
$MgSO_4$ x 7 H_2O	0.05 g
Agar	15.0 g
MiliQ	1.0 L

Protocol:

1. Dissolve components in 900 mL (starch will not fully solubilize)
2. Adjust the pH to 7.2
3. Dilute to 1 L and autoclave

DMD Media (Defined Media for *Delftia*, our own lab's protocol)

Component	**For 400 mL**	**For 500 mL**	**For 800 mL**	**For 1 L**
KH_2PO_4	0.12 g	0.15 g	0.24 g	0.30 g
$MgSO_4$	0.12 g	0.15 g	0.24 g	0.30 g
Pyruvic acid	0.64 mL	0.80 mL	1.28 mL	1.60 mL
L-glutamine	0.80 g	1.00 g	1.60 g	2.00 g
MOPS	0.80 g	1.00 g	1.60 g	2.00 g
Agar (if needed)	6.0 g	7.50 g	12.00 g	15.00 g

Protocol:

1. Add components to ~80% of the total volume MiliQ
2. Adjust the pH to 7.5 with 1.0 M NaOH (*aq)*
3. Dilute to the final total volume with MiliQ
4. Autoclave

NRRL13 Media No. 1 (following protocol from NRRL Agricultural Research Service Culture Collection: https://nrrl.ncaur.usda.gov/forms/NRRL_Medium_13.pdf)

Components:

Tryptone	5.00 g
Yeast Extract	5.00 g
K_2HPO_4	1.00 g
Glucose	1.00 g
Agar	15.0 g

Protocol:

1. Add components to 950 mL of MiliQ
2. Bring pH to 7.0
3. Dilute to a final volume of 1 L with MiliQ
4. Autoclave

ISP-3 Media (Following the protocol from Actinobase: http://actinobase.org/index.php/ISP3)

Components:

White Oats	20.0 g
Agar	18.0 g

ISP Trace Metals Mix:

$FeSO_4$ heptahydrate	0.20 g
$MnCl_2$ tetrahydrate	0.20 g
$ZnSO_4$ heptahydrate	0.20 g

Protocol:

1. Biol oats in 1 L of MiliQ for 20 minutes
2. Filter oats through cheesecloth and top up volume to 1 L
3. Adjust the pH to 7.2, add agar, and autoclave
4. Mix trace metals in 200 mL of MiliQ and filter sterilize
5. Add 1 mL of ISP Trace Metals Mix to autoclaved media before pouring plates

ISP-4 Media (Following protocol from Actinobase: http://actinobase.org/index.php/ISP4)

Components:

Soluble Starch	10.0 g
$MgSO_4$ heptahydrate	1.0 g
NaCl	1.0 g
$(NH_4)_2SO_4$	2.0 g
$CaCO_3$	2.0 g
Agar	20.0 g

ISP Trace Metals Mix:

$FeSO_4$ heptahydrate	0.20 g
$MnCl_2$ tetrahydrate	0.20 g
$ZnSO_4$ heptahydrate	0.20 g

Protocol:

1. Prepare as two separate solutions
2. Solution 1: Make a paste of soluble starch with little MiliQ, then dilute to 500 mL
3. Solution 2: Dissolve salts and 1.0 mL of ISP Trace Metals Mix in 400 mL MiliQ, adjust pH to between 7-7.4, dilute to 500 mL

Mix solutions 1 and 2, add agar, and autoclave