ISOLATION AND COMPARISON OF PLANT GROWTH PROMOTING RHIZOBACTERIA ISOLATES AND CONSORTIA IN CONFERRING DROUGHT TOLERANCE IN WHEAT SEEDLINGS

A Thesis

by

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ABSTRACT

With changes in climate, increased world population projections, and limitations to water availability, farmers are challenged to meet future food and feed demands. This study focuses on alleviation of drought stress, by way of tolerance, in a continued effort to increase crop production in industrial agriculture. The goal of this study was to isolate and identify plant growth promoting rhizobacteria (PGPR) from a soil inoculum primed for a drought tolerant host phenotype. Two novel rhizobacteria isolates, *Pseudomonas stutzeri* strain A4 and *Stenotrophomonas maltophilia* strain C3, were shown to delay the onset of drought stress phenotypes in the aboveground tissue of TAM111 wheat seedlings after ten days of water deficit. While roots inoculated with our isolated PGPR, plus another PGPR isolated in previous studies (*Enterobacter cloacae* strain 16i), were shown to have a statistically significant effect of alteration in root architecture, the comparison of the treatments (single isolate vs consortia vs control) did not reveal a treatment-specific alteration of root architecture. The second portion of this study was the *de novo* assembly of our novel PGPR isolates with a hybrid sequencing methodology based on one next generation sequencing (NGS) platform and one third generation sequencing platform. The resulting assembly and annotation of high quality draft genomes have begun to identify the presence of bacterial genes potentially related to drought tolerance, but more likely, bacterial genes will be related to a conference of overall plant health benefits under abiotic stress conditions.

DEDICATION

I dedicate this thesis to all the good people who have stuck by my side throughout this graduate school journey- both teaching me how to be a good scientist and a good human.

To my parents: may the submission of my thesis bring you fewer phone calls from me seeking support. I don't think I would have made it without you as my cheerleaders.

To my cohort and friends: I thank you for the endless comradery, advice, kickball games, beach trips, and many nights discussing life and science over a cold beer (or cider for me) at O'Bannons. Graduate school gave me a second family.

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To the man I love: let the submission of this thesis be the mark of the beginning of a new chapter of our lives. Thank you for your patience and support.

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Contributors

This work was supervised by a thesis committee consisting of Professor Young-Ki Jo, of the Department of Plant Pathology and Microbiology, Professor Heather Wilkinson of the Department of Plant Pathology and Microbiology, and Professor Rodolfo Aramayo of the Department of Biology.

The initial host mediated microbiome engineered soil inoculum for Chapter 2 was provided by Dr. Young-Ki Jo. The statistical analyses depicted in Chapter 2 were conducted in part by Dr. Young-Ki Jo of the Department of Plant Pathology and Microbiology.

All other work conducted for the thesis was completed by the student independently.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Climate change and the global water deficit

At the turn of the 21st century, over exploitation of global freshwater resources has become a major threat to the longevity of humanity. Food security is one of the factors that can greatly limit the overall well-being of society and can be directly correlated to freshwater shortages. Yet, even with this basal understanding of freshwater shortages, we are currently observing the maximum consumptive freshwater use reaching its potential breaking point (Kummu et al., 2016). To further exacerbate this trend, is the fact that the global latitudes experiencing the greatest amount of water scarcity are the most populated areas of the world (Kummu et al., 2016; Kummu & Varis, 2011). Compounding the issue of population density and per capita water use, the regions of the world with the greatest water scarcity and highest populations are also converting land for agricultural use at the highest rate globally (Kummu & Varis, 2011).

Therefore, to begin to understand the crisis we are facing with global water scarcity, we must focus on one of the largest sectors of freshwater and green water usage-- agriculture. Agricultural water usage faces both manmade and natural sources of growing water scarcity. We observe drought, aridity, desertification, and irrigation water shortages as the four limiting factors to agricultural areas undergoing water deficits (Pereira, 2005). Studies have already reported alarming physical evidence that human

activities, such as farming and agriculture, have already exceeded renewable water limits. A poignant example can be seen in six of India's important agricultural states. These regions have sustained their growing agricultural sectors (pressured by an exponentially rising birth rate) by overexploiting groundwater to a volume of water that exceeds the average annual flow of the Nile River (S. Postel, 1999). Even with the most optimistic of projections, and most efficient delivery methods, growing food demand by 2025 could require an additional 500 km^3 of irrigation water, due to increasing dependence on irrigated land and decreasing opportunities to expand rain fed crop production (S. L. Postel, 1998).

These observations lead to questions weighed down with serious implications: what global changes do we expect to see with the impending water deficit and how do we combat the seemingly inevitable?

With the world population conjecture for 2030 predicting a population increase to 8 billion people on Earth, we expect that the agricultural industry will face many concerns, with respect to increasing food production and overall food security(Smol, 2012). Parallel to the global population increase predictions for 2030, it is also estimated that global demand for cereal crops, for food and animal feed, will total 2.8 billion tons per year- a 50 percent increase from demands in 2000- without taking account of biofuel production (Timmusk et al., 2014). With an increased requirement for agricultural production globally, water availability has become the current limiting resource in agriculture, causing approximately 70 percent of all arable farmlands to have potential yield decline (Coulter, 2004). Therefore, with water availability limitations, changes in

climate, and an increasing world population in mind, farmers in both developing and developed countries will be challenged to meet future food and feed demand.

Drought has been identified as a major global challenge in the continued efforts to increase crop production in industrial agriculture (Vinocur & Altman, 2005). When studying drought, we can characterize it into the categories of meteorological, hydrological, socioeconomic, and agricultural based on its severity, frequency, and economic impact (Ngumbi & Kloepper, 2016). We will focus on the specifics of agricultural drought, which is the drought type associated with a period of declining soil moisture that results in agricultural crop failure (Ngumbi & Kloepper, 2016). Regardless of being sessile organisms who cannot physically "escape" drought conditions, plants have adapted to prolonged water deficiency through four different ecological mechanisms: escape, recovery, avoidance, and tolerance (Comas, Becker, Cruz, Byrne, & Dierig, 2013; Ngumbi & Kloepper, 2016; Sun et al., 2015; Timmusk et al., 2014; Xiong, Wang, Mao, & Koczan, 2006; Zhou & Shimizu, 2010). Drought escape is defined as a plant's tendency to end its life cycle before the onset of drought is able to take a toll on the plant's growth and reproduction (Ngumbi & Kloepper, 2016; Zhou & Shimizu, 2010). Drought recovery is defined as a plant's ability to recover its fitness and vigor once drought conditions have been naturally or artificially ameliorated (Bryla, Duniway, & Soil, 1997; Ngumbi & Kloepper, 2016). Drought avoidance is defined as a plant's ability to maintain regular water status (osmotic pressure) by mechanisms identified to be mitigation of water loss through transpiration or water uptake from the surrounding environment (Forni, Duca, & Glick, 2017; Timmusk et al., 2014; Zhou &

Shimizu, 2010). Drought tolerance, the focus of this thesis, is defined as the plant's ability to continue normal growth and metabolism regardless of the severity of drought in its environment (Comas et al., 2013; Ngumbi & Kloepper, 2016). The plant-based mechanisms that confer the phenotype of drought tolerance include differential osmotic potential, increased translation of reactive oxygen species (ROS) scavenging enzymes that locate to the cell membrane to maintain stability under dehydration due to lignification, and the accumulation of secondary metabolites specific to drought response which function to stabilize plant tissue through the water scarcity (Comas et al., 2013; Ngumbi & Kloepper, 2016).

Plant growth promoting rhizobacteria

Two major consequences to environmental stresses-like drought-are that plant growth and production are significantly lower than they would be in their absence. To be able to overcome environmental stresses, plants have the ability to modify their metabolism to induce a range of host defensive proteins for stress alleviation. Recent studies have shown that native or applied microbiota can aid in the induction of these host defense responses to abiotic stresses (Glick, Cheng, Czarny, & Duan, 2007).

Therefore, one of the most recently studied and sustainable biological approaches to cope with water deficiency in major crop production has been the application of plant-growth promoting rhizobacteria (PGPR) to crops. PGPR are naturally occurring microorganisms that can be found in the root adherent and soil that closely surrounds the root rhizosphere interface (Lugtenberg & Kamilova, 2009). When studying the

rhizoplane colonization by PGPR, colonization traits that appear to be important bacterial characteristics for overcoming abiotic and biotic stresses are chemotaxis toward root exudates, high colony growth rates in root exudates, and synthesis of amino acids (Lugtenberg & Kamilova, 2009; Van Loon, 2007). At the most basal level, we understand that these rhizobacteria are quick colonizers and have been studied in both symbiotic and non-symbiotic relationships with the plant's root rhizosphere. These root rhizosphere-based interactions are now known to give way to increases in crop fitness and resistance or alleviation of biotic and abiotic stresses. Drought tolerance is one of many phenotypes PGPR can directly confer to host plants they have colonized (Barnawal et al., 2017; Coleman-Derr & Tringe, 2014; Comas et al., 2013; Dimkpa, Weinand, & Asch, 2009; Gontia-Mishra, Sapre, Sharma, & Tiwari, 2016; Ngumbi & Kloepper, 2016; Timmusk et al., 2014; Vurukonda, Vardharajula, Shrivastava, & SkZ, 2016; Yang, Kloepper, & Ryu, 2009; Zolla, Badri, Bakker, Manter, & Vivanco, 2013)

Direct plant growth promoting rhizobacteria can be categorized into four different classes-- biofertilizers, rhizoremediators, phytostimulators, and stress controllers. Biofertilizing plant growth promoting rhizobacteria are defined as rhizobacteria that supply plants with nutrients in the absence of pathogens. Currently known examples of rhizobacteria that perform as biofertilizers would be nitrogen fixing rhizobacteria (*Rhizobium*, *Bradyrhizobium*, and *Azospirillum* spp.) and phosphate solubilizing rhizobacteria that solubilize phosphate when it is bound organically or inorganically (*Pseudomonas*, *Bacillus*, *Acinetobacter*, and *Xanthomonas* spp.) ultimately making it available for uptake by the plant (van Rhijn & Vanderleyden, 1995; Vassilev,

Vassileva, & Nikolaeva, 2006). Rhizoremediating plant growth promoting rhizobacteria are defined as pollutant degrading rhizobacteria that colonize close enough to the root exudate to use it as their primary carbon or general nutrient source during rhizoremediation. An example of a rhizoremediating PGPR would be *Pseudomonas putida* strain PCL1444, which was observed to have the ability to degrade naphthalene in the soil and stabilize the rhizosphere 100-fold better when compared to a *Pseudomonas fluorescens* strain WCS365 control (Kuiper, Bloemberg, & Lugtenberg, 2001). Phytostimulating plant growth promoting rhizobacteria are defined as rhizobacteria that produce substances, most notably the hormone auxin, which stimulate the growth of plants in the absence of pathogens. PGPR-promoted plant growth can be induced by hormones, volatiles, and cofactors excreted by the colonized beneficial rhizobacteria (Lugtenberg & Kamilova, 2009). An example of a phytostimulating PGPR that produces phytohormones would be the auxin generating *P. fluorescens* strain WCS365 which, upon root inoculation, leads to significant increases in the root weight of tryptophan-rich radishes (Kamilova, Kravchenko, Shaposhnikov, Makarova, & Lugtenberg, 2006). Examples of phytostimulating PGPR that release volatiles to promote plant growth would be *Bacillus subtilis* and *Bacillus amyloliquefaciens*. *B. subtilis* and *B. amyloliquefaciens* biosynthesize 2,3 butanediol and acetoin, causing increases in total leaf surface area in *Arabidopsis thaliana* (Ryu et al., 2003). An example of a phytostimulating PGPR that produces a cofactor to promote plant growth would be *P. fluorescens* B16. *P. fluorescens* B16 expresses pyrroloquinoline quinone (PQQ) biosynthetic genes that act as an antioxidant in plants and causes significant

increases in the fresh weight of *Cucumis sativus* spp. (Choi et al., 2008). The final class of direct plant growth promoting rhizobacteria are the stress controlling rhizobacteria. Stress controllers are defined as PGPR that produce aminocyclopropane-1-carboxylate (ACC) deaminase which relieves several forms of plant stress, such as the effects of phytopathogenic bacteria, the presence of polyaromatic hydrocarbons in the soil, the presence of heavy metals in the soil, the effects of salinization, and the effects of water deficit (Lugtenberg & Kamilova, 2009). The bacterial production of ACC deaminase can reduce the effects of plant ethylene production, which is the host plant's response to stress exposure in the rhizosphere and phyllosphere. Ethylene production in the plant will cause direct inhibition of plant growth mechanisms in both the roots and shoots of the host. The presence of ACC deaminase biosynthesizing genes is relatively common in rhizobacteria, thus creating many examples of ACC deaminase producing PGPR (E.g. *Azospirillum*, *Rhizobium*, *Agrobacterium*, *Achromobacter*, *Burkholderia*, *Ralstonia*, *Pseudomonas*, and *Enterobacter* spp.) (Barnawal et al., 2017; Glick et al., 2007; Mayak, Tirosh, & Glick, 2004; Saleem, Arshad, Hussain, & Bhatti, 2007; R. P. Singh & Jha, 2017; Xiong et al., 2006).

This study specifically focuses on two of the classes of direct plant growth promoting rhizobacteria: phytostimulators and stress controllers (Glick, Karaturovíc, & Newell, 1995; Glick, Penrose, & Li, 1998). Mechanisms reported as being associated with drought tolerance conferred by root rhizosphere manifestation of phytostimulating or stress controlling PGPR include the aforementioned production of ACC deaminase, which interferes with constitutive ethylene synthesis in the host, large-chain extracellular

polysaccharide synthesis, osmoregulation to maintain a negative water potential, transcriptional regulation of host stress responses, alteration of root architecture to enhance biomass, and production of antioxidants to scavenge or bind to reactive oxygen species (Aird et al., 2013; Barnawal et al., 2017; Dimkpa et al., 2009; Forni et al., 2017; Gontia-Mishra et al., 2016; Ngumbi & Kloepper, 2016; Osakabe, Osakabe, Shinozaki, & Tran, 2014; Timmusk et al., 2014; Vacheron et al., 2013; Vurukonda et al., 2016; Yang et al., 2009).

Assembled plant growth promoting rhizobacteria consortia

Previously, we discussed the virtues of a single rhizobacteria colony inoculum enhancing a host drought tolerance response to water deficit. The beneficial effects of single isolate PGPR have only been validated in laboratory or greenhouse studies (i.e. small-scale studies), which unfortunately does not provide a comprehensive analysis of their effect in large-scale, economically important agriculture. When single isolate PGPR have been used as plant inoculum in field studies, inconsistent results of their effectiveness have been the status quo (Barra et al., 2016). At the microbial community level in the field, we understand that the inoculated PGPR are in competition with the naturally occurring microbiota. Therefore, a new concept has begun to be implemented where the beneficial bacteria can be combined into consortia. When applied in a field setting, the small-scale community organization will stabilize the effects of competition in the natural environment.

Recent studies have observed that microbial consortia, which consist of two or more microbial isolates, have a greater complexity of dynamics with a host and can increase the robustness of the behavior we observe at the single-isolate rhizobacteria level of host-microbe interaction (Coleman-Derr & Tringe, 2014; Khan et al., 2016; Marasco et al., 2013; Mishra et al., 2016; Naveed, Mitter, Reichenauer, Wieczorek, & Sessitsch, 2014; C.-J. Wang et al., 2012; Zolla et al., 2013). While a bacterial consortium contains the same plant growth promoting (PGP) traits as a single isolate (ACC deaminase activity, solubilization of micronutrients, hormone production, EPS formation, and siderophore activity), as a community they also contain previously untapped synergistic effects (Aird et al., 2013; Pérez-Montaño et al., 2014; S. Singh & Kapoor, 1999; Swarnalakshmi et al., 2013). At a basal level, the synergistic effects have been observed to be nutrient mobilization, enhanced efficacy of host response to treatments, spatial uniformity, and stability of the populations when applied to the host. The underlying mechanisms of these effects can be attributed to community-level quorum sensing, secretion of diffusible metabolites, and secretion of enzymes- all of which enable unidirectional communication and relative cooperation in the microbial consortia (Bentley et al., 2008; Panwar, Tewari, & Nayyar, 2014; Smith, Tanouchi, & You, 2013).

In a previous study, native microbial consortia, when compared to single bacterial isolates, were found to increase stem girth, seedling height, and leaf counts in three *Lycopersicon esculentum* Mill tomato varieties (Akintokun & Taiwo, 2016). In another study, two PGPR with the ability to tolerate abiotic stress were evaluated in a

consortium to determine if, together, a stronger alleviation drought stress in chickpea (*Cicer arietinum* L.) could be observed. When the two PGPR strains were determined to be non-antagonistic to each other *in vitro*, they were applied to the plants. In a consortium, their synergistic growth enhanced the PGP attributes. The growth parameters were also observed as significantly higher in consortium, when compared to individual PGPR and control treatments (Mishra et al., 2016).

Bacterial whole genome sequencing

For this study, to begin to understand the genetic potential of drought related PGPR isolates, we needed to produce and obtain the whole bacterial genomes of putative PGPR that confer drought tolerant phenotypes to TAM 111 wheat seedlings *in planta*. Traditionally, whole bacterial genome sequencing assemblies and annotations can lead to functional predictions *in silico*, which also creates an opportunity for comparative genomic studies between ecologically similar bacterial species (Hughes Martiny & Field, 2005). Within drought-specific studies, whole genome sequencing and draft genome assembly can help identify genes putatively involved in beneficial plant growth promoting (PGP) traits (Z. Wang et al., 2018).

In the past decade, high throughput sequencing has been improved upon in such a way that benchtop scientists can cheaply implement the sequencing techniques into their "toolbox" when studying microbiota like bacteria (Edwards & Holt, 2013). New sequencing technologies have emerged and challenged the long-standing status-quo of Sanger Sequencing, coining the term "second generation sequencing". Second

generation sequencing technologies like pyrosequencing, sequencing by synthesis, and ion semiconductor sequencing have been developed, as well as a third generation of single-molecule platforms (Aird et al., 2013; Bentley et al., 2008; Korlach et al., 2010; Pushkarev, Neff, & Quake, 2009; Quainoo et al., 2017; Rothberg et al., 2011; Wick, Judd, & Holt, 2019). Robust competition between manufacturers has created a high-stakes environment of improvement, which has allowed sequencing capabilities to improve on almost a six-month basis (Loman et al., 2012).

In 2011, Oxford Nanopore Technologies (ONT), announced the launch of a new third-generation single-molecule sequencing platform that would challenge the existing market. The promised output from ONT was read-lengths orders of magnitude longer than existing sequencing technologies, a low cost-per-base, and a small, portable sequencing machine (Mikheyev & Tin, 2014). With this technology, reads for bacterial genomes can be delivered in a lab setting in hours rather than months. Regardless, with the diversification of sequencing platforms, has come an overwhelming, and disorganized plethora of relevant analytical tools supported by specific sequencing read types. The novice bioinformatician can often struggle to identify the correct methodology for their sequencing platform, assembly, annotation, and analysis.

When trying to resolve complex bacterial genomes, opting to use the long reads provided by ONT MinION sequencing allows for more sequencing accuracy in the highly repetitive regions of the unknown bacterial isolate genome (Bouchez, Baines, Guillot, & Brisse, 2018; Goodwin et al., 2015; Loman, Quick, & Simpson, 2015; Mikheyev & Tin, 2014; Wick et al., 2019). Also, when compared to the price of more

traditional Illumina sequencing, if a lab already has access to ONT products, the cost of sequencing per sample is almost equal, or with native barcoding, even less when using the ONT technology "in house." However, with every emerging sequencing platform comes pitfalls. While providing long, single sequence reads, Oxford Nanopore technology also creates high sequence error rates, when compared to traditional Illumina sequencing or Pacific Biosciences sequencing (Quainoo et al., 2017).

Therefore, this study implemented a hybrid approach to bacterial whole genome sequencing that combined reads produced from one Illumina sequencing run and two Oxford Nanopore MinION sequencing runs (George et al., 2017; Risse et al., 2015). The rationale of using these two sequencing platforms in tandem to resolve *de novo* assembly of whole bacterial genomes is, that the long reads from the MinION will help resolve the ordering and highly repetitive regions of the smaller pair-wise, fragmented reads produced by the Illumina sequencing.

Thesis Goals

The overall goal of my research is to identify a method to begin alleviation of drought stress (by way of tolerance) in economically important cereal crops, through the constructs of root inoculations of PGPR consortia.

Objective I, Part A

Using a rhizosphere microbiome, from a previous project completed with collaborators, that has undergone host mediated microbiome engineering for drought

tolerance, we will isolate putative plant growth-promoting rhizobacteria (PGPR). In the isolation process, semi-selective media will be used and the most morphologically dissimilar single colonies will be preserved for further investigation.

Objective I, Part B

Using wheat (TAM111, hard red winter wheat variety) as a phytometer, we will use a rapid screening method to compare out putative PGPR for strong phenotypic performance in conferring drought tolerance in a growth chamber setting. Using observed physiological response ratings from our rapid screening rating system, the putative PGPR will be differentiated from our less beneficial rhizobacteria under osmotic stress conditions.

Objective II

Using the single colony isolates of putative PGPR that were screened for their ability to confer a drought tolerant phenotype, we will combine the isolates into consortia and run plant assays again. We will observe physiological response with our rapid screening rating system for above ground tissue. We will also analyze the below ground tissue for physiological differences compared to plants inoculated with single isolates and our negative controls. These plant assays will be performed as an effort to elucidate the mechanism(s) of action for PGPR conferring drought tolerance in consortia.

Objective III

Using a hybrid sequencing method, we will sequence the PGPR isolates that were used in our consortia plant assays. Assembling the whole draft genomes will allow us to taxonomically identify the isolates to strain level specificity. The assembled bacterial draft genomes will also allow us to investigate the presence or absence of genes associated with plant drought stress alleviation through annotation techniques.

Hypotheses

Hypothesis I

Out of the total number of culturable rhizobacteria isolated in *Objective I*, *Part A*, only 15 individual isolates will be genetically distinct from one another. Since the individual isolates were cultured from microbiomes selected to improve plant drought tolerance, each isolate will improve plant drought tolerance when compared to the controls in the single isolate plant assays of *Objective I*, *Part B*.

Hypothesis II

Since the individual rhizobacteria were isolated from the same or similar original microbiome inocula, selected based on the ability to confer a drought tolerant phenotype to the plant, there will be several combinations of consortia that improve plant drought tolerance when compared with the individual isolates and controls in *Objective II*.

Hypothesis III

With the use of ONT and Illumina sequencing technology, we will be able to assemble and resolve the draft genomes for each individual PGPR isolate. With the draft genomes, we will also be able to confirm the presence of bacterial genes related to drought tolerance.

CHAPTER II

SCREENING AND IDENTIFICTION OF PGPR ASSOCIATED WITH DROUGHT TOLERANCE AND ASSEMBLED BACTERIAL CONSORTIA THAT ENHANCE EFFECT OF DROUGHT TOLERANCE IN ROOT RHIZOSPHERE

Introduction

Cereal production in North America, Europe, and Australasia suffer the most from droughts that cause substantial yield deficit during harvest (Lesk, Rowhani, & Ramankutty, 2016; Pereira, 2005). Historically, developed nations suffer the most from water deficit due to the lack of diversification in cropping, which has resulted in large monocultures being grown on the same field plots over multiple seasons (Gornall et al., 2010; Lobell et al., 2014). More recent droughts (1985-2007) have had a severe impact on cereal crop production in North America when compared to the previous documented seasons from 1964 to 1984 (Gornall et al., 2010; Lesk et al., 2016; Lobell et al., 2014; Sheffield, Wood, & Roderick, 2012). Therefore, cold winter wheat, an economically important cereal grain, was chosen as the model host organism for this study. To eliminate the variable of genotype affecting any objective outcomes, one genotype of cold winter wheat (TAM 111), was used for all accompanying studies in this chapter.

Previously studied methods to mediate drought stress in wheat have revolved around conventional plant breeding techniques to identify drought resistant varieties for field application, also called drought resistant germplasm (Fischer & Maurer, 1978) Other groups have studied methods for the genetic engineering of transgenic wheat crops

to modify germplasm resistance to water deficit, which has concurrently revealed many difficulties in actual field application (Fischer & Maurer, 1978). Both of these remedies overlook the ecological community of the rhizosphere environment supporting plant growth and reproduction, which has shown that plant-associated microbes can influence a plant's response to abiotic stresses. As soil microbes rapidly adjust to water deficit conditions causing decreases osmotic pressure in the rhizosphere, they also provide resources to the plant roots by releasing solutes and phytohormones to mediate the belowground stress.

The first objective of this study was to isolate single PGPR capable of rapidly, and robustly colonizing wheat seedling rhizospheres and mediating drought stress individually. When combined in consortia, the second objective was to evaluate an additive effect of increased drought tolerance with the PGPR combinations.

The rationale behind bioprospecting potential putative PGPR from the seventh generation of the drought tolerant host mediated microbiome engineered soils (HMME) (Jochum, McWilliams, Pierson, & Jo, 2019) was to increase the likelihood of selecting a soil source hosting an ecological community containing PGPR that would be able to mediate drought stress. The host-mediated microbiome engineering, microbial, starting material underwent seven rounds of pervasive water stress conditions and provided an observable drought tolerance in wheat seedlings (Jochum, McWilliams, Pierson, & Jo, 2019).

In this study, we planned to isolate plant-growth-promoting rhizobacteria (PGPR) that are capable of increasing plant growth, development, and resistance under

drought stress. Previous screening approaches in the Jo Lab discovered bacterial strains *Bacillus* sp. (12D6) and *Enterobacter* sp. (16i) that confer bacterially mediated host drought tolerance to wheat seedlings (*Triticum aestivum* subsp. *aestivum* TAM 111) (Jochum et al., 2019). 12D6 and 16i improved root phenotypes to resist drought stress. Introduction of these PGPR to the host rhizosphere improved the drought tolerance phenotypes including specific root length (SRL), specific root surface area (SRA), leaf dry matter content (LDMC), root tissue density (RTD), and the amount of root branching/forks compared to the untreated control. Those PGPR were used as a successful bacterial agent in remediating water deficiency during the onset of seedling drought in wheat. We predicted that the new PGPR isolated in this study, individually, would directly or indirectly produce signals that alter root system architecture for increased water uptake surface area exploration, while also producing a biofilm that adds the humectant properties of the rhizosphere, increased water retention, and prevention of host root desiccation.

As stated previously, we predicted that when applied to the root rhizosphere, the individual PGPR isolates will show a superior above ground phenotype, when compared to the negative controls. All previously explained expectations of individual PGPR treatments remain true for our expectations of the bacterial consortia treatments that were applied to the host root rhizosphere. This prediction is derived from the understanding that the consortia treatments are equimolar portions of the identified, individual PGPR isolates, with the same starting colony concentrations as the individual treatments. However, instead of expecting only a superior aboveground phenotype in the

plant drought assays, when compared to the negative controls, we also expected that the consortia treatments would express superior aboveground phenotypes when compared to the single isolate PGPR treatments, in our rapid rating screening method. At the root rhizosphere level, we predicted that the drought tolerance conveyed by treatment specific root architecture would be greater with the consortia treated plants when compared to single isolated PGPR treatments and the negative control.

Materials and Methodology

Host-mediated microbiome engineering for drought tolerance phenotype

In a previous collaboration (Jochum, McWilliams, Niu & Jo, 2019), twenty-five samples of wild bermudagrass were sampled using a soil corer during the summer of 2016 in the semi-arid environment of El Paso, Texas. Sampling sites were a mix of different non-commercial sites including ranches, highway medians, parks, and roadsides. Bermudagrass samples were shipped to the Jo Lab green house in College Station, Texas. Upon arrival, each sample core was sub-divided into four 2-inch diameter cores of Bermuda grass and associated rhizosphere. The cores were then individually planted with small amounts of sterile Metro-Mix 900 for stability, and exposed to varying degrees of water deficit in the greenhouse. The water treatments for the cores consisted of watering to field capacity every other day (control), once a week, or no watering. The onset of drought symptoms for the bermudagrass were monitored and recorded based on several phenotypes: wilting, leaf curling, necrosis of the leaf, and lodging of the stem. The top 5 “no water” cores that were able to withstand the onset of

water deficit were then used for host-mediated microbiome engineering (HMME), artificial selection of microbes inhabiting the root rhizosphere interface. Next, cold winter wheat seeds (*Triticum aestivum* subsp. *aestivum* cultivar TAM111) (Lazar et al., 2004) were transferred into 12 cm x 10 cm circular pots filled with 400 grams of sterilized Metro-Mix 900 potting mix and with amalgamated "engineered" rhizosphere mix from the original, Top 5, bermudagrass inoculum. Five germinated seeds were sown for each treatment per pot. Germinated seeds were watered to field capacity on Day 0 and were then cultivated without any watering at 25°C using fluorescent lights emitting approximately 300 $\mu Em^{-2}s^{-1}$ with a 12:12 hour light/dark cycle. Each round of microbiome engineering was concluded at the point in time which 90 percent of the pots (45 out of 50) exhibited the aforementioned signs of drought stress. The remaining 5 pot's rhizospheres that showed the phenotype of drought tolerance were selected to be the inoculum for the next round of engineering.

After seven rounds of selection, host-mediated microbiome engineering enabled the plants to endure the duress of water deficit by an additional 5 days when compared to the original Bermuda grass inoculum trials. By the seventh round, however, there was no longer an increase or decrease in the duration of time the plants could endure water deficit, so that was considered the final generation. The root adherent soil from the 'Top 5' pots in round seven were separated from the roots and aboveground tissue, then collected for further experiments. These are the rhizosphere microbiomes used in the present investigations for Objective I and II.

Bioprospecting rhizobacteria from root adherent soil

Following the collection of the root adherent soil from the seventh round of host mediated microbiome engineering, 5 mL of the amalgamated soil from the 'Top 5' microbiomes in round seven were placed in 50 mL Falcon tubes. The root-adherent soil was homogenized in 10mL of 0.1 M phosphate buffer saline (PBS) for 1 minute, followed by a serial dilution with 0.1 M PBS. An aliquot of 100 µL from the 10^{-3}, 10^{-5}, and 10^{-7} dilution series was plated onto Luria-Bertani (LB) agar amended with cycloheximide (20 mg L^{-1}) as well as King's B agar amended with cycloheximide (20 mg L^{-1}). The plates were then incubated at 30-32°C and checked every 4 hours for single colony formation. Once the first single bacterial colony formations were established at 16 hours, all morphologically distinct colonies (a total of 64) were transferred and four-quadrant streaked to either a new LB agar plate or King's B plate, depending on the original media on which they were grown. The plates were then incubated at 30-32°C for 16 hours. One single colony from each isolation was transferred into a test tube containing 4 mL of LB broth and shook at 120 rpm overnight (16 h) at 30-32°C. The overnight cultures were transferred into 40% sterile glycerol stocks and stored at -80°C for long term cryopreservation.

Plant assays for drought tolerance with single rhizobacteria isolates

TAM111 wheat seeds (*Triticum aestivum* subsp. *aestivum* cultivar TAM111) (Lazar et al., 2004) were surface sterilized in 10% sodium hypochlorite + 1 M Tween20 for 10 min of gentle stirring followed by 10 washes in sterile ddH_2O. Seeds that floated

to the top of the sterilization step, seeds that were cracked, and seeds that had an observed disease phenotype were promptly discarded. The surface sterilized seeds that did not get disposed of were then germinated on sterile filter paper at 25°C in an incubator for 24h. Germinated seeds were then transferred into "cone-tainer" pots (SC10 cells have a cell diameter of 1.5", a depth of 8.25" and a volume of 164 mL or 10 cu. in) filled with 150 grams of sterile Metro-Mix 900 potting mix. Three seeds were sown for each initial treatment, with one seed per cone-tainer. Wheat seedlings were watered to field capacity every day and cultivated for 7 days at 25°C, using fluorescent light emitting approximately 300 $\mu Em^{-2}s^{-1}$ with a 12:12 hour light/dark cycle. Isolated rhizobacteria were inoculated onto the wheat seedlings after 7 days of seedling growth. LB overnight cultures (2 mL) of each bacterial isolate were centrifuged into a pellet at 5000 rpm for 5 minutes at 25°C. The LB supernatant was poured off and the pellet was suspended in a volume equal to the supernatant poured off of PBST. 100 µL of the bacterial solution containing 30-50 x 10^{10} CFU/mL was applied to the base of the seedling and beginning of the rhizosphere using a sterile pipet tip. Seedlings then underwent 10 days of water deficit, by withholding all watering for the 10 days. After 10 days of water deficit, the plants were graded on a scale of 1, 2, 3, 4, and 5 (1- complete lodging, severe leaf curling, noticeable stunting, and permanent wilting; 2- complete lodging, minor leaf curling, noticeable wilting; 3- no lodging, moderate leaf drooping on both leaves; 4- slight wilt of flag leaf; 5- no symptoms). The described Rapid Rating System can be found in *Appendix A*. This initial plant assay was repeated two more times, and the scores for the treatments were averaged for each biological replicate and

compared to the untreated control. Ten individually isolated putative PGPR treatments out of the 64 were then preliminarily identified in the following step.

Preliminary identification of PGPR

The nucleotide sequences of the best 10 PGPR isolates showing drought tolerance phenotypes with the host were determined initially by 16S/23S rDNA amplification and sequencing. Genomic DNA (gDNA) from the isolates was extracted using the Phire Plant Direct PCR kit (Thermo Fisher Scientific). One single colony from each bacterial isolate was suspended in dilution buffer and used as template DNA for the rapid reaction. Using polymerase chain reaction (PCR), the bacterial isolate's conserved 16S and 23S ribosomal ribonucleic acid (RNA) subunit and internal transcribed spacer (ITS) regions were amplified. Primers (with a starting concentration of 10μM with Promega Nuclease Free Water) used for the amplification of the 16S to 23S region were 805F/pA (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse p23/SR01 (5'-GGCTGCTTCTAAGCCAAC-3') (Dinesh et al., 2015). The 40 μL PCR reaction was carried out in the thermocycler (Applied Biosciences Thermocycler 2720) and programmed for 1 cycle of 2 min at 95°C, 40 cycles of 30 sec at 95°C, 30 sec at 52.0°C, and 3 min at 72°C, followed by one cycle for 60 sec at 72°C and then 4°C until retrieval from the thermocycler. To confirm PCR amplification, we used gel electrophoresis. 40 μL of PCR product in a 1.5% agarose gel impregnated with 1μL of ethidium bromide (20mg/100mL) was submersed in TAE buffer for 45 minutes at 750 mV. A 4 μL aliquot of the O'GeneRuler Express DNA Ladder (Thermo Fisher Scientific) was run on the gel

in parallel with the PCR product to compare the band sizes with the predicted product measuring 3000bp. Image visualization of the expected 3kb construct was confirmed using a UV illuminator and Alphaimager 2000 v5.5 (Alpha Innotech Corporation). When the amplification was successful, the amplified region from the 16S and 23S rRNA amplicon (approximately 3kb in length) was excised from the agarose gel and purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research). The purified amplicons were then sent to Eton Biosciences for Sanger Sequencing. All Sanger single-pass sequencing was done with user-supplied primers at 10µM concentrations. With successful sequencing, 16S/23S forward read results were taxonomically identified at the genus level through the SILVA "High Quality Ribosomal RNA" database SINA (v1.2.11) (Quast et al., 2013) that classified the reads based on the least common ancestor (LCA) method, based on 16S SSU taxonomy hosted by SILVA. In the event that the SILVA database did not provide a match with sufficient detail, the sequences also went through a second taxonomic identification at the genus specific level through the NCBI Nucleotide Basic Local Alignment Search Tool (BLASTN). When the results showed that several of the samples were the same genus or even species, ClustalΩ (European Molecular Biology Laboratory- European Bioinformatics Institute) was used to create a phylogenetic tree and choose the bacteria that showed the greatest amount of phylogenetic diversity to be part of the final set of isolated rhizobacteria used in later objectives.

Plant assays for drought tolerance with assembled bacterial consortia

TAM111 wheat seeds (*Triticum aestivum* subsp. *aestivum* cultivar TAM111) (Lazar et al., 2004) were surface sterilized in 10% sodium hypochlorite + 1 M Tween20 for 10 min of gentle stirring followed by 10 washes in sterile ddH_2O. Seeds that floated to the top of the sterilization step, seeds that were cracked, and seeds that had an observed disease phenotype were promptly discarded. The surface sterilized seeds that did not get disposed of were then germinated on sterile filter paper at 25°C in an incubator for 24h. Germinated seeds were transferred into "cone-tainer" pots (SC10 cells have a cell diameter of 1.5", a depth of 8.25" and a volume of 164 mL or 10 cu. in) filled with 150 grams of sterile Metro-Mix 900 potting mix. Three seeds were sown for each initial treatment, with one seed per cone-tainer. Wheat seedlings were watered to field capacity every day and cultivated for 7 days at 25°C using fluorescent light emitting approximately 300 $\mu Em^{-2}s^{-1}$ with a 12:12 hour light/dark cycle. Bacterial consortia, containing every pairing of the two identified putative PGPR (A4 and C3) with the 16i internal positive control (Michael D. Jochum et al., 2019) were inoculated onto the wheat seedlings after 7 days of seedling growth. LB overnight (16hr) cultures (2 mL) of each bacterial isolate (A4, C3, and 16i) were centrifuged into a pellet at 5000 rpm for 5 minutes at 25°C. The LB supernatant was poured off and the pellet was suspended in an equal volume of PBST. 100 µL of a 1:1 bacterial consortia solution containing 30-50 x 10^{10} CFU per 1 mL was applied to the base of the seedling and beginning of the rhizosphere using a sterile pipet tip. Seedlings underwent 10 days of water deficit, by withholding all watering for the 10 days. After 10 days of water deficit, the aboveground

plant phenotype was measured as per the aforementioned grading scale (Appendix A). This plant assay was repeated nine more times, and the scores for the treatments were averaged for each biological replicate (n=10). The scores of the biological replicates were recorded to observe the above ground phenotype of the assembled consortia when compared to the single isolates and the negative control treatment. The additive effect of increasing the consortia population one member at a time was observed when applying the aforementioned grading scale. This initial consortia assay was repeated with 10 biological replicates per treatment with all plants harvested after 7 days of growth and then the 10 days water deficit, at a total of 17 days post-germination. Detritus and soil were removed from the roots with ddH_2O and a 0.5 mm mesh sieve. The harvested root and shoot tissue were then prepped for downstream analysis.

Measuring the rhizosphere drought tolerance phenotype

Harvested root and shoot tissues were saturated with reverse osmosis H_20 overnight in wet germination paper at 6°C, in preparation for WinRHIZO analysis. Before analysis, washed roots were separated from their shoot tissue and placed on a flatbed scanner (EPSON, Perfection V-750). The seedling roots were positioned with 5 roots per tray [20cm x 30cm], covered with just enough reverse osmosis H_20 to create a layer of cohesion with the scanner and allow roots to be spread out but not overlap one another. The roots were scanned into .jpeg images that were incorporated into WinRHIZO Arabidopsis 2017a (Regent Instruments Inc.) software. The WinRHIZO program analyzed the scanned images and generated estimates of total root length (cm),

projected root area (cm^2), root surface area (cm^2), average root diameter (mm), total root volume (cm^3), total number of root tips, total number of root forks, and total number of root crossings.

Statistical analysis

The PGPR consortia plant assays were conducted in a random block design, with 8 treatments containing 10 biological replicates, and every treatment block position randomized for each replication. The initial consortia assays contained 8 experimental replications for the rapid rating assay. An additional 2 experimental replications were conducted to obtain rhizosphere data used in the WinRHIZO analysis. Aboveground plant phenotype data was analyzed using LR Statistics for Type I Analysis (SAS version 9.3 software, SAS Institute Inc, Cary, NC). Root phenotype data from WinRHIZO results were analyzed using analysis of variance (ANOVA) (SAS version 9.3 software, SAS Institute Inc, Cary, NC). Pairwise comparisons between the treatments were conducted using Fisher's least significant difference (LSD) test [$P < 0.05$].

Results

From the microbial inoculum procured in the seventh round of host mediated microbiome engineering for drought tolerance in wheat seedlings (Jochum, McWilliams, Niu & Jo, 2019), 64 single bacterial colonies were isolated in the first steps of the experiment. Initial isolation was based on several criteria previously documented as characteristics of putative PGPR, to increase the likelihood of reducing the number of

Table 1 Initial identification of PGPR. Sanger Sequencing on the high conserved 16S to 23S RRNA region of the bacteria

Sample ID	QC	Length	NCBI BLAST®	Percent Identity	Accession
A4	53	1989	*Pseudomonas stutzeri* strain 28a24	97%	CP007441.1
B11	48	1149	*Pseudomonas stutzeri* strain ICS4 16S ribosomal RNA gene	99%	JQ995477.1
C3	53	2050	*Stenotrophomonas maltophilia* strain SKK55 chromosome	98%	CP040433.1
C4	51	1011	*Stenotrophomonas sp.* pho chromosome	99%	CP029759.1
C9	42	930	*Pseudomonas hibiscicola* strain R8-737 16S ribosomal RNA gene	99%	JQ659977.1
D3	53	2026	*Stenotrophomonas maltophilia* strain SKK55 chromosome	98%	CP040433.1
D5	53	796	*Bacterium* strain BS0681 16S ribosomal RNA gene	99%	MK823869.1
D9	47	1156	*Bacterium* strain BS1574 16S ribosomal RNA gene	98%	MK824762.1
E5	51	996	*Stenotrophomonas maltophilia* strain S-3 16S ribosomal RNA gene	99%	JX868559.1
E10	51	636	*Stenotrophomonas sp.* strain MR68 16S ribosomal RNA gene	99%	MG674364.1
F2	27	2012	*Stenotrophomonas maltophilia* strain SKK55 chromosome	98%	CP040433.1

colonies that would not possess the desired characteristics. The 64 bacterial isolates were purposefully chosen due to their ability to colonize quickly (under 18 hours of incubation). Secondly, they were isolated based on the simple conditions of growth on LB and King's B agar at temperatures between 30-32 °C. Qualitative assessment of host performance across replicate experiments suggested that out of the 64 isolates tested in single inoculant plant assays 11 putative PGPR alleviated drought stress symptoms when compared to the controls. Results from Sanger sequencing of the 16S and 23S rRNA region and NCBI BLAST® query identified the rhizobacteria as *Pseudomonas* sp. for isolate A4, *Pseudomonas* sp. for isolate B11, *Stenotrophomonas* sp. for isolate C3, *Stenotrophomonas* sp. for isolate C4, *Pseudomonas* sp. for isolate C9, *Stenotrophomonas* sp. for isolate D3, unknown *Bacterium* strain for isolate D5, unknown *Bacterium* strain for isolate D9, *Stenotrophomonas* sp. for isolate E5, *Stenotrophomonas* sp. for isolate E10, and *Stenotrophomonas* sp. for isolate F2 (Table 1).

After phylogenetic analysis, two isolates with large phylogenetic differences were used in the consortia plant assays (A4 and C3) with another known PGPR identified in a previous study (16i) (Jochum et al., 2019). In the consortia assays, qualitative assessment of the aboveground host tissue (Figure 7, Appendix B), via the rapid rating system, showed that single isolate PGPR alleviated drought stress symptoms when compared to all consortia combinations and the control (Figure 1,2).

Quantitative results from the below ground tissue analysis (Figure 3) from a one-way ANOVA revealed varying results with the tested dependent variables (total root length (cm), projected root area (cm^2), root surface area (cm^2), average root diameter (mm), total root volume (cm^3), total number of root tips, total number of root forks, and total number of root crossings) (Table 2). Further analysis with Dunnett's Test will be implemented to account for the simultaneous comparing of each treatment with a control group.

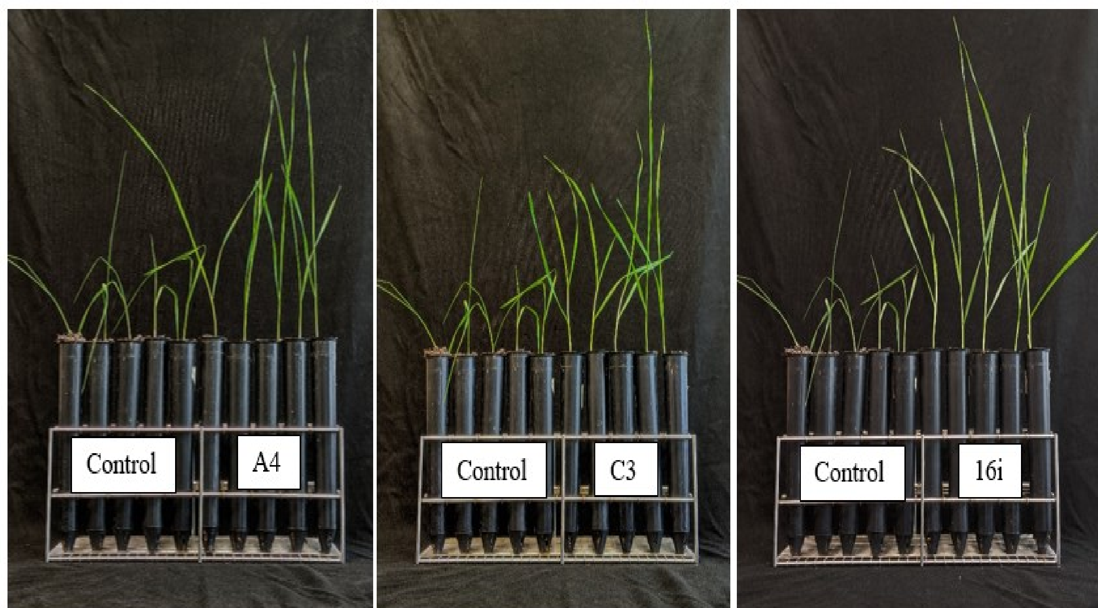

Figure 1 Wheat seedlings inoculated with single PGPR. Wheat seedlings treated with single plant growth promoting rhizobacteria isolates A4 *(Pseudomonas* sp.), C3 (*Stenotrophomonas* sp.), and 16i (*Enterobacter* sp.), compared to the control after 10 days of continuous water deficit.

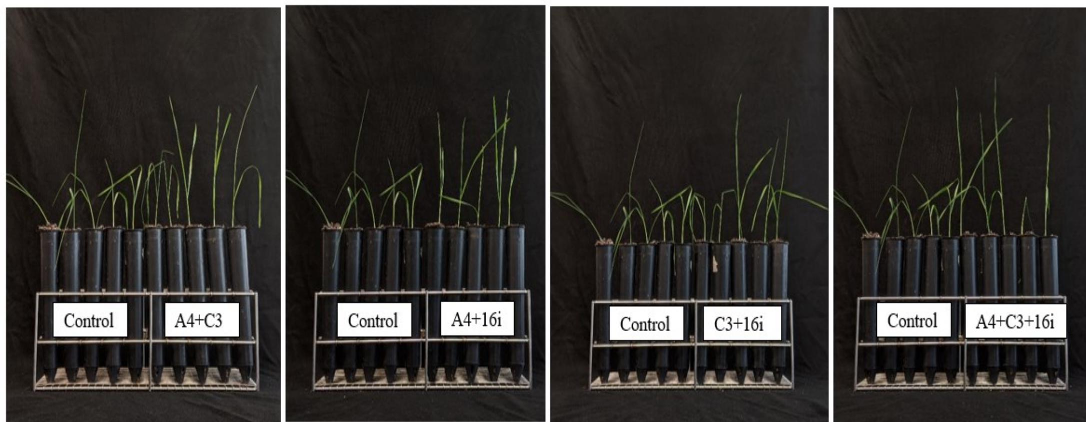

Figure 2 Wheat seedlings inoculated with PGPR consortia. Wheat seedlings treated with consortia combinations of A4 (*Pseudomonas* sp.), C3 (*Stenotrophomonas* sp.), and 16i (*Enterobacter* sp.) after 10 days of continuous water deficit, compared to the control.

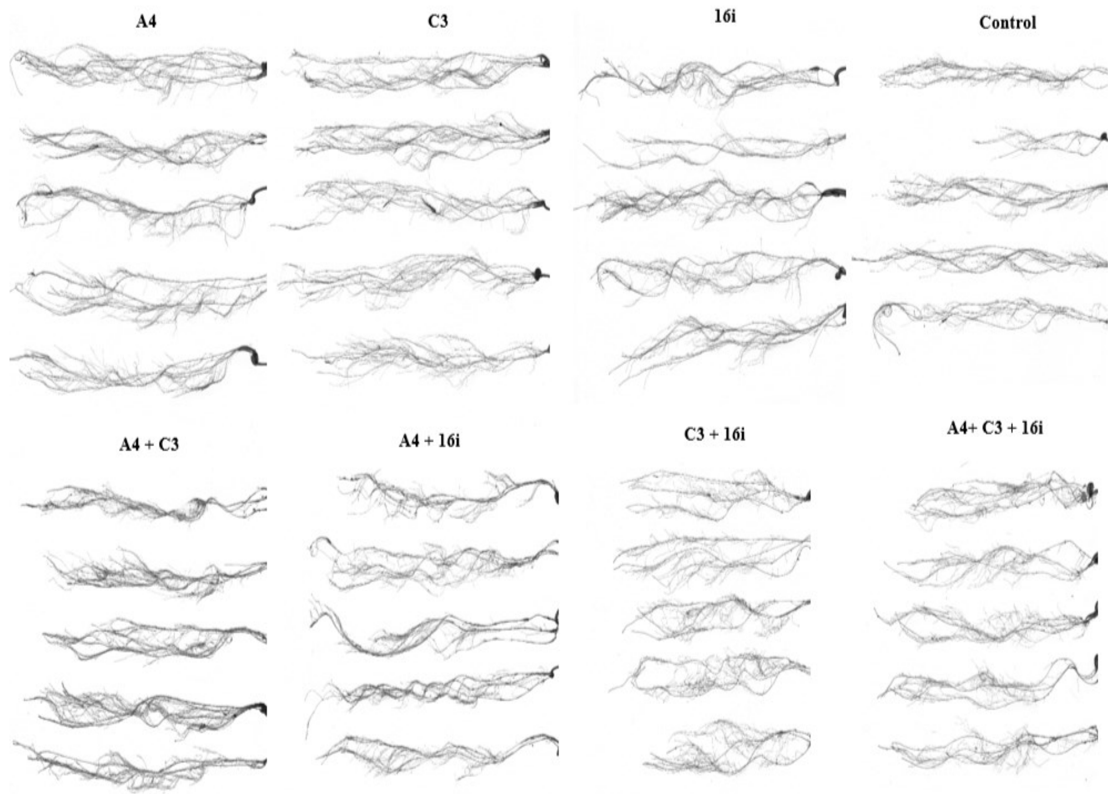

Figure 3 Root system architecture of inoculated wheat seedlings. Root system architecture of wheat seedlings treated with the control (**top far right**), *Pseudomonas* sp. A4 (**top far left**), *Stenotrophomonas* sp. C3 (**top center left**), *Enterobacter* sp. 16i **(top center right)**, *Pseudomonas* sp. A4 and *Stenotrophomonas* sp. C3 consortia (**bottom far left**), *Pseudomonas* sp. A4 and *Enterobacter* sp. 16i (**bottom center left**), *Stenotrophomonas* sp. C3 and *Enterobacter* sp. 16i (**bottom center right**), and *Pseudomonas* sp. A4 plus *Stenotrophomonas* sp. C3 plus *Enterobacter* sp. 16i consortia **(bottom far right)**, after 10 days of water deficit post inoculation.

Table 2 Analysis of variance of PGPR treatments. Analysis of variance (ANOVA) for the effect of plant growth promoting rhizobacteria (PGPR) treatment on wheat seedlings following a 10- day water deficit post inoculation

	Trial I				*Trial II*			
Dependent Variables	**df**	**Means Squared**	**F**	**Pr>F**	**df**	**Means Squared**	**F**	**Pr>F**
Average Root Length	7	13966.0758	2.70	0.0054	7	6291.1348	3.60	0.0022
Average Root Surface Area	7	43.3439	1.75	0.1103	7	27.4540	2.80	0.0123
Average Diameter	7	0.0009	2.41	0.0281	7	0.0018	6.12	<0.0001
Root Tips	7	101994.5430	1.64	0.1379	7	38502.3980	2.04	0.0615
Root Forks	7	861786.0900	1.93	0.0778	7	302663.1980	3.16	0.0057

In our first trial, treatment of seedlings with *Pseudomonas sp.* (A4) contributed to greater total root length when compared to the treatment combination of *Stenotrophomonas* sp. (C3) and *Enterobacter* sp. (16i), but not significantly greater than any other treatment, including the control. In the second trial, the seedling treatment with *Stenotrophomonas* sp. (C3) contributed to a greater total root length when compared to the treatment combination of *Stenotrophomonas* sp. (C3) and *Enterobacter* sp. (16i), but not significantly greater than any other treatment, including the control. In trial one and two, the single isolates did not consistently significantly improve total root length when compared to the controls or the other treatments, excluding the combination of C3 and 16i (Table 3).

In our first trial, the seedling treatments with A4, C3, 16i, the combination of all three isolates, and the negative control contributed to a significantly smaller average root diameter, when compared to the treatment combination of C3 and 16i. In the second trial, single isolates A4 and C3 contributed to a smaller root diameter when compared to

the treatment combination of all three PGPR, C3 and 16i, A4 and C3, A4 and 16i, and the control (Table 3).

Table 3 Pairwise comparison using Fisher's LSD test. Pairwise comparisons using Fisher's LSD t-test (n=10) of wheat seedling root architecture with single PGPR and consortia treatments analyzed with WinRHIZO software.

Trial Number	Treatment	Root length (cm)	Root surface area (cm2)	Average diameter	Number of root tips	Number of root forks
Trial I	A4	343.18	20.686	0.1921C	752.50	2431.5
	C3	311.07	18.067	0.1830C	704.20	1985.2
	16i	288.73	17.997	0.2008C	652.20	2059.9
	A4 + C3	259.80	22.931	0.2913A	702.80	2601.0
	A4 + 16i	289.21	21.695	0.2428B	871.40	2590.7
	C3 + 16i	212.53	18.932	0.2919A	341.56	1884.3
	A4 + C3 + 16i	299.35	18.832	0.1995C	656.10	2228.7
	control	273.97	16.799	0.1949C	531.30	1913.7
Trial II	A4	178.58	11.610	0.2058	444.40	999.7
	C3	214.82	14.429	0.2127	573.30	1226.8
	16i	175.85	12.026	0.2171	563.70	1116.3
	A4 + C3	192.53	13.783	0.2293	494.50	1236.5
	A4 + 16i	179.71	13.118	0.2348	516.60	1235.9
	C3 + 16i	127.69	9.252	0.2280	367.50	755.9
	A4 + C3 + 16i	195.36	13.546	0.2206	524.00	1258.7
	control	173.39	13.505	0.2482	472.30	1209.1

Means in the same column of each trial with the same letter are not significantly different at P=0.05

As a consequence of average root diameter, in our first trial, the seedling treatment of A4, C3, 16i, the combination of all three isolates, and the negative control, all contributed to a significantly smaller root volume in the seedling rhizosphere when compared to all other treatments. Subsequently, in our second trial, the seedling treatments with A4, and the combination of C3 and 16i, all contributed to a significantly smaller average root volume in the seedling rhizosphere when compared to all other treatments in this trial.

Discussion

These experiments use a bioprospecting pipeline that has previously been developed for having effectively screened PGPR isolated from the root rhizosphere interface, one of which was used as a positive control for the single isolate plant assays and consortia plant assays in this study *(Enterobacter* sp. 16i) (Jochum et al., 2019). However, certain modifications to the accepted pipeline were made to enhance selection for rhizobacteria that were part of a root-adherent rhizosphere microbiome primed for drought tolerance. By starting the rhizobacterial isolations from soil inoculum that had undergone seven generations of host mediated microbiome engineering (Jochum, McWilliams, Niu & Jo, 2019) and an initial rapid screening assay to quickly identify the desired above ground traits of drought tolerant plants, we effectively narrowed down the bacterial isolates from 64 putative PGPR to 12 putative PGPR. Sanger sequencing identified that some of the remaining 12 rhizobacteria isolated from bioprospecting and the initial screening were closely related (to species level specificity) to previously documented plant growth promoting rhizobacteria, when compared to the top taxon hits from a tblastn search using the rhizobacteria's 16S-23S rRNA region with the NCBI BLAST® database.

When treated with single isolate PGPR, the qualitative plant performance suggests that wheat seedlings undergo a delay of onset drought symptoms when compared to the consortia and the control treatments, however further below ground phenotype analysis showed that we cannot conclude that this observable phenotype is

associated with changes in the root system architecture of the single isolate treated wheat seedlings.

When compared to the observable, positive, above ground phenotype of single PGPR isolates in the consortia assays, the measurable below ground phenotype data suggests there are much more complicated relationships in the root rhizosphere interface occurring. The host's response to PGPR is complex and the results of this study challenge previously documented cases of PGPR inoculations and the host's response to abiotic stresses, like water deficit.

We were able to conclude from our analysis of the below ground tissue, through analysis of variance, that the single isolate PGPR and the consortia created from the PGPR contributed to the production of greater average root length in both *Trial I* and *Trial II,* greater average root surface area in *Trial II*, lower average root diameter in *Trial I* and *Trial II*, greater root forking in *Trial II*, all when compared to the experimental control. However, when comparisons were run between treatments to differentiate the treatment specific influence on the root rhizosphere architecture, no clear understanding of treatment specific host phenotype was elucidated. The only consistent trend we saw in the comparison of treatment specific influence via Fisher's LSD t-test, was the overall underperformance of the consortia combination C3+16i. When compared to all other treatments, including the control, C3+16i had the lowest total root length in *Trial I* and *Trial II*, the lowest number of root tips in *Trial I* and *Trial II*, and the lowest number for root forks in *Trial I* and *Trial II*., and a significantly larger average root diameter in *Trial I*. Based on both the quantitative below ground tissue analysis and the qualitative above

ground tissue screening, we substantiate that antagonist behavior occurred between the *Stenotrophomonas* sp. C3 and *Enterobacter* sp. 16i *in planta*.

The canonical understanding of PGPR rests around the idea that changes in specific aspects of the root rhizosphere are positively correlated to the overall improvement of host stress tolerance to water deficit and drought tolerance created by maintaining plant productivity over the stress period. Previous studies have elaborated that enhanced root architecture by way of PGPR application, specifically elongated total root length, increased total root surface area, decreased average root diameter, and increased total number of root tips. All these host phenotypes allow increase in water uptake by the roots with an expanded rhizosphere area which allows for increase water and nutrient resource scavenging (Barnawal et al., 2017; Dinesh et al., 2015; Gontia-Mishra et al., 2016; Jochum et al., 2019; Ngumbi & Kloepper, 2016; Pérez-Montaño et al., 2014; Porcel, Zamarreño, García-Mina, & Aroca, 2014; Vacheron et al., 2013; Vurukonda et al., 2016; Yang et al., 2009).

Though known and documented PGPR, the *Pseudomonas* sp., *Stenotrophomonas* sp., and *Enterobacter* sp. isolated in our study did not demonstrate enhanced below ground phenotypes, and yet, still produced a greater shoot biomass when compared to the consortia and control treatments. Perhaps, rather than enhancing the below ground root rhizosphere architecture, these rhizobacteria are interacting with the host in more subtle ways that promote overall greater host fitness with the onset of abiotic stresses, like water deficit.

Previous studies of *Pseudomonas stutzeri* strains, the same genus and species as our isolate A4, have shown under normal and oxygen-limiting environments, that P. stutzeri strains can be used as a bio-fertilizer to activate N_2 fixation (Pham et al., 2017; Yan et al., 2010). *P. stutzeri* can assimilate molecular nitrogen from the atmosphere into a host-soluble form of nitrogen that can be taken up by roots. It was shown, with *P. stutzeri* strain A15, that in oxygen limiting soil, the inoculation of this bacterial treatment could increase the above ground biomass of rice (*Sativa oryzae*) by three fold, when compared to the mutant, non-nitrogen fixing strain *(P. stutzeri nifD* mutant)(Pham et al., 2017; Yan et al., 2010).

Previous studies of *Stenotrophomonas maltophilia* strains, the same genus and species as our isolated C3, have shown under normal environmental conditions that *S. maltophilia* strains increase phosphate solubilization in the root rhizosphere of sugar cane (*Saccharun* spp.) *S. maltophilia* strain CA158 and CA79 solubilized phosphate in higher values than other bacterial PGPR treatments, increasing plant height, stem diameters, total number of tillers, leaf area, and overall aboveground biomass(Berg, 2009; González, Victoria, & Merino, 2015; Lamizadeh, Enayatizamir, & Motamedi, 2016; R. P. Singh & Jha, 2017). Another study even showed *S. maltophilia* strains played a role as a biocontrol agent for the root-dampening pathogen *Pythium ultimum* (R. P. Singh & Jha, 2017).

Previous studies of *Enterobacter cloacae* strains, the same genus and species as the isolate 16i, have shown *E. cloacae* to produce ACC deaminase under various abiotic and biotic stresses in the root rhizosphere. While ACC deaminase production has

historically been understood as a rhizobacterial response to osmotic stress in the root rhizosphere, as a regulator of ACC (a precursor to ethylene) levels in the plant root exudate, further studies have shown drought stress to not be the only environmental condition eliciting this response (Glick et al., 2007; Glick, Liu, Ghosh, & Dumbroff, 1997; Gontia-Mishra et al., 2016; Mayak et al., 2004). Production of ACC deaminase by *E. cloacae* and other PGPR in the root rhizosphere can also be a response to salinity stress, waterlogging stress, temperature stress, pathogenicity stress, heavy metal stress, organic contaminant stress, air pollutant stress, and root nodulation promotion (Saleem et al., 2007).

With the results of our study and previous documentation of the other PGPR traits, our three PGPR isolates may have different mechanisms to promote plant drought tolerance other than improved root architecture.

CHAPTER III

HYBRID SEQUENCING AND ASSEMBLY OF WHOLE BACTERIAL GENOMES FROM ISOLATED PGPR USED IN ASSEMBLED BACTERIAL CONSORTIA

Introduction

In genomics, or ‘omics, era the technology to rapidly sequence high quality draft genomes is constantly evolving and gaining precision (Marx, 2013). Due to the highly motivated “arms race” biotechnology companies are locked in to produce better sequencing technology, scientists evolved their techniques beyond first-generation Sanger Sequencing to include high-throughput sequencing methods, coining the term ‘second generation sequencing’ (Loman et al., 2012).

After the first whole bacterial genome was published in 1995 (Fleischmann et al., 1995), technology quickly evolved to improve the ability for scientists to have reads longer than the small amplicon sizes Sanger sequencing produced. Second generation sequencing technology pairs PCR and fluorescence to be able to detect the identity of nucleotides binding to the small fragments of template DNA that have been ligated to adapters on the solid-surface flow cell (Huptas, Scherer, & Wenning, 2016). This process allows for the documentation of DNA sequencing clusters, ultimately creating the reads that will be processed into FASTQ output from the platform. The most commonly used second generation sequencing methods currently are Illumina sequencing platforms (HiSeq, MiSeq, and iSeq) (Huptas et al., 2016; Quainoo et al., 2017). When sequencing high quality genomic DNA, these platforms produce paired-

end short reads ranging from 150-250 base pairs long. These short reads have low base calling error rates that can produce millions of reads, depending on the length of time the platform is run in the sequencing center. The quality of the reads produced, when trimmed and filtered, allow for scientists to pursue *de novo* assembly of the microbial genomes or high continuity mapping to reference genomes, depending on the purpose of the project.

However, the limitations of second-generation high throughput sequencing of complicated prokaryotic and eukaryotic genomes have also been documented. Short reads cannot always resolve the high repetitive (GC rich) regions of prokaryotic or eukaryotic genomes, nor can they always resolve genetic evolutionary events that take the form of plasmid formation, transposable elements, or methylation sites (De Maio et al., 2019; Kaas, Leekitcharoenphon, Aarestrup, & Lund, 2014; Loman et al., 2012; McKernan et al., 2009; Quainoo et al., 2017). Without consistently being able to solve these intricate aspects of prokaryotic or eukaryotic genomes, complete, high-quality draft genomes cannot be assembled with the accuracy required for current publication standards.

The biotechnology industry's answer to this problem has been the development of third generation, single-cell sequencing platforms such as Pacific Biosciences (PACBIO) sequencing and Oxford Nanopore Technology (ONT) (Loman et al., 2012; Loman et al., 2015; Quainoo et al., 2017). When compared to the prices of Illumina sequencing platforms (approximately $78.00-$8900.00 per gigabase[Gbase], platform dependent), single molecule, real-time sequencing platforms have reduced the cost per

Gbase in the case of ONT MinION sequencing, or competitively match the costs in the case of PACBIO Sequel system (Quainoo et al., 2017). However, the competitive or reduced platform prices for the reads comes at quality disadvantages when compared to Illumina sequencing platforms. Both PACBIO and ONT platforms have increased error rates between 12-15% per base, unlike Illumina's approximate 0.1% error rate in greater than 80% of the total bases called. Also, the number of reads are reduced in a standard 24 hour sequencing run with PACBIO platforms or ONT platforms, when compared to Illumina platforms (Bouchez et al., 2018; Goodwin et al., 2015; Laver et al., 2015; Mikheyev & Tin, 2014; Quainoo et al., 2017; Quick, Quinlan, & Loman, 2014; Wick et al., 2019). Regardless of the reduction in quality and total number of captured reads, scientists are still adopting this technology due to the real-time output and cost differentials.

In our study we opted to adopt two sequencing platforms, also known as hybrid sequencing, to draft the three bacterial genomes used in the aforementioned assembled bacterial consortia studies: *Pseudomonas* sp. A4, *Stenotrophomonas* sp. C3, and *Enterobacter* sp. 16i. Based on cost, we chose Illumina iSeq sequencing to produce 150 base pair paired-end reads and ONT MinION sequencing (which was already present in our lab) to produce the single-end long reads necessary to resolve the highly repetitive, and even palindromic, regions of bacterial genomes (Bouchez et al., 2018; De Maio et al., 2019; George et al., 2017; Risse et al., 2015). We assessed each method's performance using standard assembly quality metrics such as total number of contiguous reads (contigs), N50, max contig length, and mean contig length.

The hybrid sequencing data ascertained from the whole genome sequencing will help identify (to strain level specificity) key bacterial isolates involved in conferring a drought tolerant phenotype in cold winter wheat. The data retrieved from this study will begin to help us elucidate potential synergisms between bacterial species that can be used for alleviation of drought stress in wheat and other economically important cereal crops.

Materials and Methodology

Bacterial isolate growth and DNA isolation

Preserved bacterial isolates were cultured (in a 40% glycerol stock) from cryopreservation at -80°C and four-quadrant streaked onto individual LB agar plates amended with cycloheximide (20 mg L^{-1}). The plates were then incubated at 30-32°C for 16 hours. One single colony from each isolation was transferred into a test tube containing 4 mL of LB broth and shook at 120 rpm overnight (16 h) at 30-32°C. LB overnight cultures (2 mL) of each bacterial isolate were centrifuged into a pellet at 5000 rpm for 5 minutes at 25°C. The LB supernatant was poured off and the pellet was suspended in a volume equal to the supernatant poured off of PBST. Genomic DNA (gDNA) was isolated and purified from the bacterial isolates using the ZymoBIOMICS DNA Miniprep Kit, SKU D4300 (Zymo Research Corporation) following the manufacturer's instructions. Purified gDNA concentrations were determined with quantification using a Nanodrop 2000/2000c (Thermo Fisher Scientific, NanoDrop Technologies Inc., Wilmington, DE). Presence of high quality, high molecular weight

gDNA was subsequently verified by agarose gel electrophoresis with a 1.5% agarose gel impregnated with 1µL of ethidium bromide (20mg/100mL). One purified sample from each individual bacterial species was sent to the Genomics and Bioinformatics Center at Texas A&M University (http://www.txgen.tamu.edu/) for Illumina Sequencing. One purified sample from each individual bacterial species was kept in house (Jo Lab, Department of Plant Pathology and Microbiology, Texas A&M University) for ONT MinION sequencing.

Illumina library construction and sequencing

Illumina library preparation followed the Low Sample Protocol for TruSeq(R) DNA Sample Preparation (Illumina, Inc.), according to the manufacturer's specifications. The purified gDNA samples were fragmented, end-repaired, 3' end-adenylated, and then ligated to paired-end adapters. The ligated products then went through gel purification and PCR amplification. Before the library-prepared samples could be run in the iSeq, their concentrations were normalized and the samples were pooled. Sample libraries were sequenced using Illumina iSeq (Illumina, Inc.) technology. Sequence cluster identification, initial quality pre-filtering, base calling and uncertainty assessment were done in real time using iSeq software version 1.3.0.1098, iSeq server version 1.3.0.18, and Recipe Fragment version 6.1.4 software with default parameter settings. Sequencer .bcl basecall files were formatted into FASTQ files using bcl2fastq 2.20 script demux_illumina.

MinION library construction and sequencing

All steps for library construction were performed according to Oxford Nanopore Technologies' (ONT, UK) protocols with minor modifications to improve upon the length of the fragments, in an attempt to receive the longest fragments possible with the fewest opportunities for DNA shearing. Following fragmentation, end-repair and dA-tailing (New England Biolabs, USA) were performed in a combined reaction followed by clean-up with 0.4x Agencourt AMPure XP beads (Beckman Coulter, UK). All incubation periods during clean-up steps were modified to 60 minutes. Unique barcodes were ligated onto each sample using Blunt/TA Ligase Master Mix (New England Biolabs, USA) and cleaned in 0.4x Agencourt AMPure XP beads. Barcoded samples were then pooled in equimolar amounts and then barcoding adapters were ligated using Quick T4 DNA Ligase (New England Biolabs, USA). Samples were subsequently run on the R10 flow cell with nanopores suitable for 1D experiments using the Ligation Sequencing Kit (SQK-LSK109). The sequencing run concluded at 20 hours with an average temperature of 33°C and -205mV.

Trimming and filtering read data

After sequencing ONT fast5 read files were base called using Albacore v2.0.2 (https://github.com/Albacore/albacore) with barcode demultiplexing and fastq output. Adapter sequences were trimmed with Porechop v0.2.2 (https://github.com/rrwick/Porechop). Remaining long reads were then error corrected using CANU v1.5 (https://github.com/marbl/canu), however all reads were filtered out

due to average Phred scores of approximately 8 for each individual isolate. These results did not become applied to downstream analysis. Illumina sequencing fastq read files were trimmed and filtered using NGS QC Toolkit v2.2.3 (Patel & Jain, 2012). The fastQC v0.10.1 algorithm was used for visual confirmation of high quality (trimmed and filtered) 150 base pair paired-end reads. Reads passed the filter if at least 80% of their nucleotides had a Phred quality greater than 20. Reads that lost their forward or reverse counterpart during filtering were packaged together into a 'singleton' FASTQ file for downstream assembly. Identification of the bacterial FASTQ files were completed with NCBI BLAST®.

Hybrid sequencing assembly

SPAdes v3.12.0 (https://github.com/ablab/spades) was used to perform sequence assembly, using a built-in read-error correction by setting the '-careful' option. With the read lengths being at or under 149 base pairs, the k-mer combination was set to 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127. The first assembly contained the forward R1.fasta from the Illumina sequencing, reverse R2.fasta, and the packaged 'singles' S1.fasta. The produced contig01.fasta file from the first round of sequence assembly was fed back into the SPAdes tool for a bootstrapping method that then applies R1.fasta, R2.fasta, S1.fasta, and contig01.fasta, to reduce overall contig number and to increase the N50 of the draft genome assembly. This produced contigs02.fasta. The next round of bootstrapping incorporated R1.fasta, R2.fasta, S1.fasta, contigs01.fasta, contigs02.fasta, and the

filtered ONT long reads that had previously been converted to FASTA files after running through FASTQGroomer (Galaxy Tool Version 1.0.4) and the FASTX_toolkit FASTQ_to_FASTA (Galaxy Tool Version 1.0.0). For all three sequenced and assembled bacterial genomes, the assembly with the lowest number of contigs and the highest N50 was defined as optimal for downstream visualization and annotation.

Aligning and visualizing assembled genomes with MAUVE

After draft genome assembly, MAUVE, or Multiple Alignment of Conserved Genomic Sequence with Rearrangements (http://darlinglab.org/mauve/user-guide/introduction.html) (Rissman et al., 2009) was used to align the three bacterial draft genomes to closely relative reference genomes (Table 4) and reorder the contigs. ProgressiveMAUVE was used to build genome alignments based on a guide tree algorithm. MAUVE Contig Mover (MCM) was used to reorder the draft genome contigs based on the reference genome assembly order.

Table 4 Reference genomes used for alignment and visualization. Reference genome strains used in ProgressiveMAUVE alignment and MCM contig reordering

Draft Genome ID	Reference Genome	NCBI Accession	G+C%
A4	Pseudomonas stutzeri strain 28a24 chromosome, complete genome	NZ_CP007441.1	60.6
C3	Stenotrophomonas maltophilia K279a, complete sequence	NC_010943.1	66.3
16i	Enterobacter cloacae subsp. cloacae ATCC 13047 chromosome, complete genome	NC_014121.1	54.8

Draft genome annotation

Preassembled and reordered FASTA files of the three bacterial genomes were

annotated using Prokka (https://github.com/tseemann/prokka)(Seemann, 2014) rapid prokaryotic genome annotation. The annotation output was in the form .gff, .gbk, .fna, .faa, .ffn, .sqn, .fsa, .tbl, .err, .log, .txt, and .tsv files for downstream analysis.

Results

After extracting high quality gDNA and sending the samples to their respective sequencing platforms, sequencing commenced. From the ONT MinION sequencing, A4 had 762 reads analyzed, with an average Phred score of 8.90, a total read yield of 5.6 megabases(Mbases), and an average read length of 7288 base pairs. Through the EPI2ME 'What's in my Pot' (WIMP) bacterial identification platform, A4 was confirmed as a *Pseudomonas stutzeri* strain. With ONT MinION sequencing, C3 had 2125 reads analyzed, with an average Phred score of 8.80, a total read yield of 16.5 Mbases, and an average read length of 7764 base pairs. Through the EPI2ME WIMP bacterial identification platform, C3 was confirmed as *Stenotrophomonas maltophilia* strain. With ONT MinION sequencing, 16i had 2737 reads analyzed, with an average Phred score of 8.99, a total read yield of 18.3 Mbases, and an average read length of 6698 base pairs. Through the EPI2ME WIMP bacterial identification platform, 16i was confirmed as an *Enterobacter cloacae* strain (Table 5).

Table 5 Oxford Nanopore MinION sequencing run read quality output. ONT Nanopore MinION sequencing run read quality output (specific to ONT) and EPI2ME WIMP bacterial identification.

Sequence ID	ONT WIMP Identification	Reads Analyzed	Total Read Yield	Average Phred Score	Average Read Length
A4	*Pseudomonas stutzeri* strain	762	5.6 Mbases	8.90	7288
C3	*Stenotrophomonas maltophilia* strain	2125	16.5 Mbases	8.80	7764
16i	*Enterobacter cloacae* strain	2737	18.3 Mbases	8.99	6698

From Illumina iSeq sequencing, A4 had a total read yield of 1.5 Mbases, an average Phred score of 36, and average read lengths of 149 base pairs. Using NCBI BLAST™, A4 was confirmed as a previously unidentified strain of *Pseudomonas stutzeri.* With Illumina iSeq sequencing, C3 had a total read yield of 1.4 Mbases, an average Phred score of 36, and average read lengths of 145-149 base pairs. Using NCBI BLAST™, C3 was confirmed as a previously unidentified strain of *Stenotrophomonas maltophilia.* With Illumina iSeq sequencing, 16i had a total read yield of 1.4 Mbases, an average Phred score of 36, and average read lengths of 145-149 base pairs. Using NCBI BLAST™, 16i was confirmed as a previously unidentified strain of *Enterobacter cloacae* (Table 6).

Table 6 Illumina iSeq sequencing run read quality output. Illumina iSeq sequencing run read quality output and NCBI BLAST® bacterial identification.

Sequence ID	NCBI BLAST® Identification	Total Read Yield	Average Phred Score	Average Read Length
A4	*Pseudomonas stutzeri* strain	1457027	36	149
C3	*Stenotrophomonas maltophilia* strain	1363602	36	145-149
16i	*Enterobacter cloacae* strain	1360451	36	145-149

After analyzing the initial read quality output, trimming adapters, and filtering the reads for only the high-quality output, the reads were assembled with SPAdes v.3.12.0 using the aforementioned bootstrapping method. After one round of assembly, *Enterobacter cloacae* strain 16i, had a total contig count of 3,411, with a GC content of 50.6%, and an average contig length (N50) of 309,680 base pairs. After the second round of bootstrapping, the number of total contigs for 16i decreased to 3,373, the GC content decreased to 50.5%, and the average contig length (N50) increased to 313,144 base pairs. After the final round of bootstrapping (which included the addition of the ONT MinION reads), the total number of contigs decreased to 3,313, the GC content stabilized at 50.5%, and the average contig length (N50) increased to 650,581base pairs (Figure 4).

1	2
GC_content	50.6
len_N50	309680
len_max	1029249
len_mean	1869
len_median	415
len_min	128
num_A	1569643
num_C	1617631
num_G	1607614
num_N	0
num_T	1580951
num_bp	6375839
num_bp_not_N	6375839
num_seq	3411

1	2
GC_content	50.5
len_N50	313144
len_max	1029249
len_mean	1899
len_median	414
len_min	128
num_A	1579063
num_C	1623738
num_G	1612552
num_N	0
num_T	1591508
num_bp	6406861
num_bp_not_N	6406861
num_seq	3373

1	2
GC_content	50.5
len_N50	650581
len_max	1308419
len_mean	1952
len_median	416
len_min	128
num_A	1591019
num_C	1661790
num_G	1607088
num_N	0
num_T	1607783
num_bp	6467680
num_bp_not_N	6467680
num_seq	3313

Figure 4 FASTAstatistics output from 16i sequence assembly. FASTAstatistics output from the first round of bootstrapping using the SPAdes assembly algorithm with Illumina forward, reverse, and single reads from the sequencing run of *Enterobacter cloacae* strain 16i as input **(left).** FASTAstatistics output from the second round of bootstrapping using the SPAdes assembly algorithm with Illumina forward, reverse, single, and contigs01.fasta reads from the sequencing run of *Enterobacter cloacae* strain 16i as input **(center).** FASTAstatistics output from the thirds and final round of bootstrapping using the SPAdes assembly algorithm with Illumina forward, reverse, single, contigs01.fasta, contigs02.fasta, and ONT MinION reads from the sequencing run of *Enterobacter cloacae* strain 16i as input **(right).**

1	2
GC_content	62.8
len_N50	1318278
len_max	2172235
len_mean	170521
len_median	371
len_min	128
num_A	914140
num_C	1540697
num_G	1563218
num_N	0
num_T	927069
num_bp	4945124
num_bp_not_N	4945124
num_seq	29

1	2
GC_content	62.8
len_N50	1698668
len_max	2172235
len_mean	190186
len_median	371
len_min	128
num_A	914078
num_C	1540601
num_G	1563164
num_N	0
num_T	926997
num_bp	4944840
num_bp_not_N	4944840
num_seq	26

1	2
GC_content	62.8
len_N50	2172024
len_max	2197654
len_mean	260207
len_median	360
len_min	128
num_A	920802
num_C	1551749
num_G	1551350
num_N	0
num_T	920034
num_bp	4943935
num_bp_not_N	4943935
num_seq	19

Figure 5 FASTAstatistics output from A4 sequence assembly. FASTAstatistics output from the first round of bootstrapping using the SPAdes assembly algorithm with Illumina forward, reverse, and single reads from the sequencing run of *Pseudomonas stutzeri* strain A4 as input **(left).** FASTAstatistics output from the second round of bootstrapping using the SPAdes assembly algorithm with Illumina forward, reverse, single, and contigs01.fasta reads from the sequencing run of *Pseudomonas stutzeri* strain A4 as input **(center).** FASTAstatistics output from the thirds and final round of bootstrapping using the SPAdes assembly algorithm with Illumina forward, reverse, single, contigs01.fasta, contigs02.fasta, and ONT MinION reads from the sequencing run of *Pseudomonas stutzeri* strain A4 as input **(right).**

After one round of assembly, *Pseudomonas stutzeri* strain A4, had a total contig count of 29, with a GC content of 62.8%, and an average contig length (N50) of 1,318,278 base pairs. After the second round of bootstrapping, the number of total contigs for A4 decreased to 26, the GC content remained at 62.8%, and the average contig length (N50) increased to 1,698,668 base pairs. After the final round of bootstrapping (which included the addition of the ONT MinION reads), the total number of contigs decreased to 19, the GC content remained at 62.8% and the average contig length (N50) increased to 2,172,024 base pairs (Figure 5).

1	2
GC_content	49.7
len_N50	479763
len_max	2116059
len_mean	71464
len_median	608
len_min	128
num_A	2545120
num_C	2365347
num_G	2536364
num_N	0
num_T	2415201
num_bp	9862032
num_bp_not_N	9862032
num_seq	138

1	2
GC_content	49.7
len_N50	479763
len_max	2118845
len_mean	71465
len_median	640
len_min	128
num_A	2473127
num_C	2474270
num_G	2427550
num_N	0
num_T	2487343
num_bp	9862290
num_bp_not_N	9862290
num_seq	138

1	2
GC_content	49.7
len_N50	479640
len_max	2117578
len_mean	189300
len_median	76339
len_min	1224
num_A	2471023
num_C	2420595
num_G	2470560
num_N	0
num_T	2481464
num_bp	9843642
num_bp_not_N	9843642
num_seq	52

Figure 6 FASTAstatistics output from C3 sequence assembly. FASTAstatistics output from the first round of bootstrapping using the SPAdes assembly algorithm with Illumina forward, reverse, and single reads from the sequencing run of *Stenotrophomonas maltophilia* strain C3 as input **(left).** FASTAstatistics output from the second round of bootstrapping using the SPAdes assembly algorithm with Illumina forward, reverse, single, and contigs01.fasta reads from the sequencing run of *Stenotrophomonas maltophilia* strain C3 as input **(center).** FASTAstatistics output from the thirds and final round of bootstrapping using the SPAdes assembly algorithm with Illumina forward, reverse, single, contigs01.fasta, contigs02.fasta, and ONT MinION reads from the sequencing run of *Stenotrophomonas maltophilia* strain C3 as input **(right).**

After one round of assembly, *Stenotrophomonas maltophilia* strain C3, had a total contig count of 138, with a GC content of 49.7%, and an average contig length (N50) of 479,763 base pairs. After the second round of bootstrapping, the number of total contigs for C3 remained 138, the GC content remained at 49.7%, and the average contig length (N50) remained at 479,763 base pairs. After the final round of bootstrapping (which included the addition of the ONT MinION reads), the total number of contigs decreased to 52, the GC content remained at 49.7% and the average contig length (N50) decreased to 479,640 base pairs (Figure 6).

After assembly, the final draft of each organisms draft contigs were input into MAUVE for visualization and contig reordering. Draft genomes (Table 4) were compared to their closest familial relative's annotated genomes for alignment, and then for reordering (Figure 7-9). In the output from ProgressiveMAUVE alignments, each colored block, or locally collinear block, represents a contig with sequence regions that align to parts of the draft genome that are homologous without internal rearrangements. To denote this relationship, the locally collinear blocks that match in the alignments are connected by vertical lines to one another. When compared to the reference genomes, all draft genomes had high similarity with several deviations from the reference genome. In the case of *Enterobacter cloacae* strain 16i, there were 27 locally collinear blocks in common with the reference genome. For *Pseudomonas stutzeri* strain A4, there were 21 locally collinear blocks in common with the reference genome. For *Stenotrophomonas maltophilia* strain C3, there were 38 locally collinear blocks in common with the reference genome. Also, in the case of *Stenotrophomonas maltophilia* strain C3, an entire new half of the genome was present in the draft genome when compared to the reference genome (NC_010943.1) that had 0 locally collinear blocks in common with the reference genome. When all the reference genomes were compared to each other in a three-way alignment, there were large differences between all three, with minimal locally collinear blocks expressing homogeny at the highly conserved regions expected of prokaryotic genomes. When all the draft genomes were compared in a three-way alignment (Figure 11), the presence of locally collinear blocks increased substantially

when compared to the three-way alignment of the reference genomes (Figure 10). All draft genome contigs were reordered based on their initial alignment to their respective reference genomes (Figure 7-9) for downstream annotation applications.

After reordering the draft contigs, the annotation was performed for all three organisms. Through the annotation platform, all three draft genomes had their species identification reconfirmed as *Pseudomonas stutzeri* (A4), *Stenotrophomonas maltophilia* (C3), and *Enterobacter cloacae* (16i).

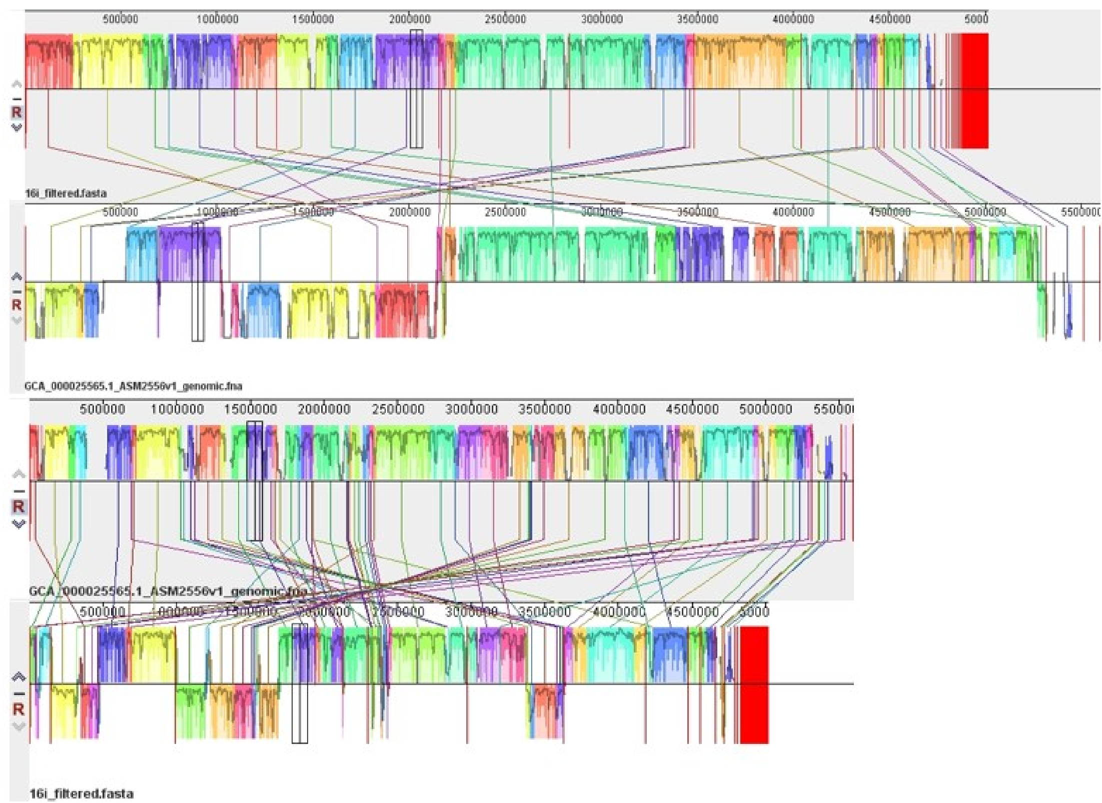

Figure 7 ProgressiveMAUVE alignment and reordering of 16i. Alignment of *Enterobacter cloacae* strain 16i to the closest familial reference genome (NCBI ASM2556v1) using MAUVE ProgressiveMAUVE alignment algorithm **(top).** Reordering of *Enterobacter cloacae* strain 16i draft genome using MAUVE reorder_contigs command with the closest familial reference genome (NCBI ASM2556v1) **(bottom).**

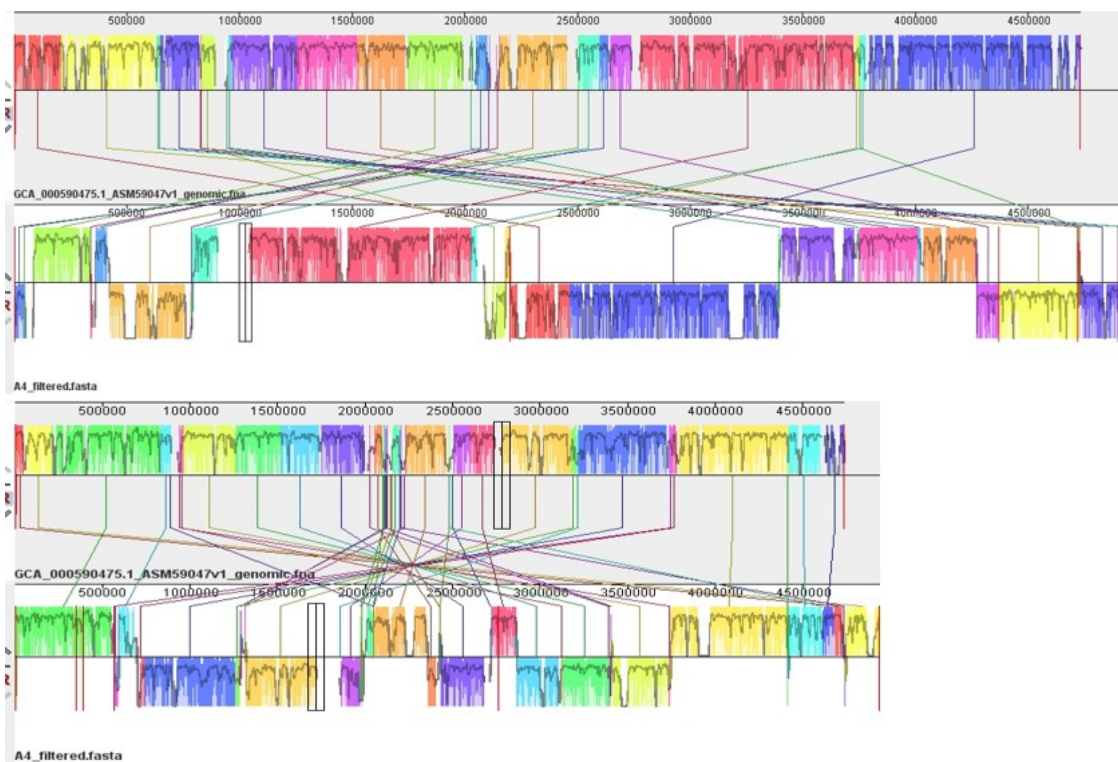

Figure 8 ProgressiveMAUVE alignment and reordering of A4. Alignment of *Pseudomonas stutzeri* strain A4 to the closest familial reference genome (NCBI ASM59047v1) using MAUVE ProgressiveMAUVE alignment algorithm **(top).** Reordering of *Pseudomonas stutzeri* strain A4 draft genome using MAUVE reorder_contigs command with the closest familial reference genome (NCBI ASM59047v1) **(bottom).**

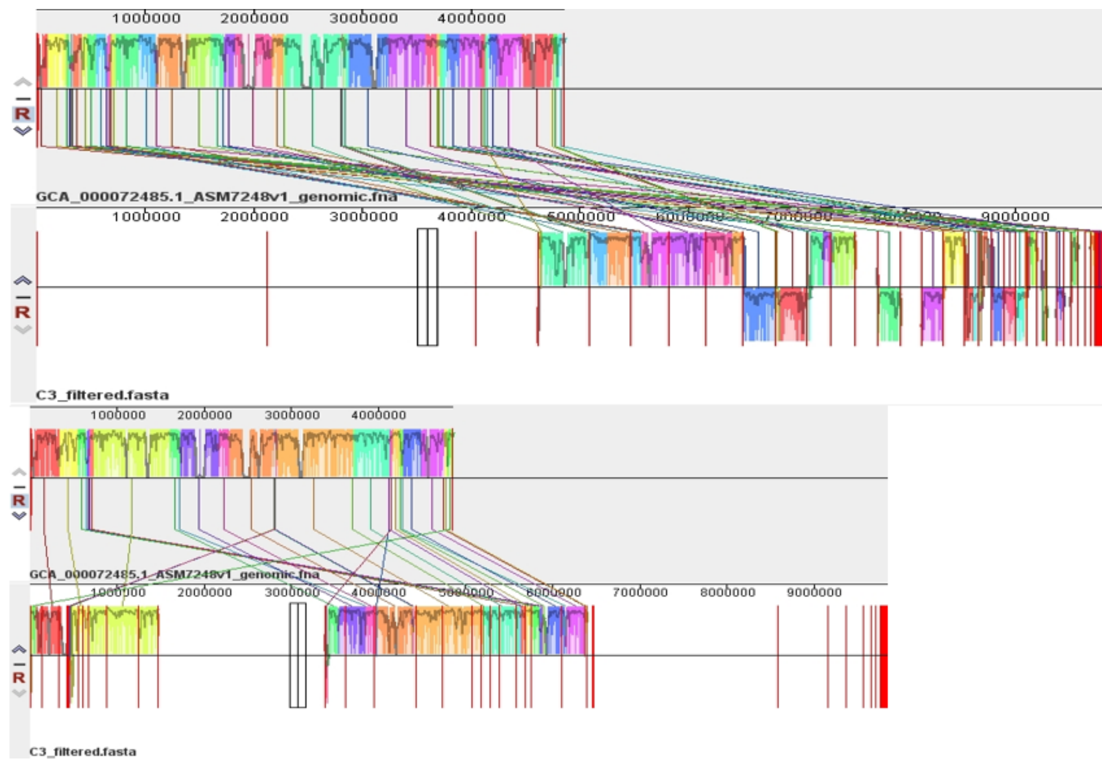

Figure 9 ProgressiveMAUVE alignment and reordering of C3. Alignment of *Stenotrophomonas maltophilia* strain C3 to the closest familial reference genome (NCBI ASM7248v1) using MAUVE ProgressiveMAUVE alignment algorithm **(top).** Reordering of *Stenotrophomonas maltophilia* strain C3 draft genome using MAUVE reorder_contigs command with the closest familial reference genome (NCBI ASM7248v1) **(bottom).**

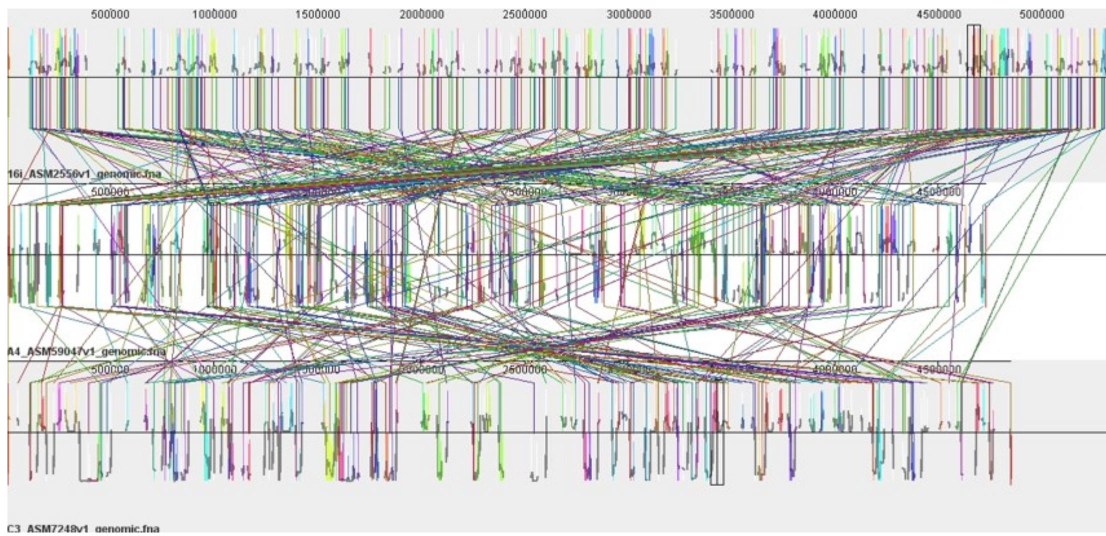

Figure 10 ProgressiveMAUVE alignment of three reference genomes. ProgressiveMAUVE three way alignment of the *Enterobacter cloacae* reference genome (ASM2256v1), the *Pseudomonas stutzeri* reference genome (ASM59047v1), and the *Stenotrophomonas maltophilia* reference genome (ASM7248v1) chosen as the closest familiar complete genomes to our three putative PGPR. The three-way alignment shows very little similarity among the three genomes due to their order level dissimilarity.

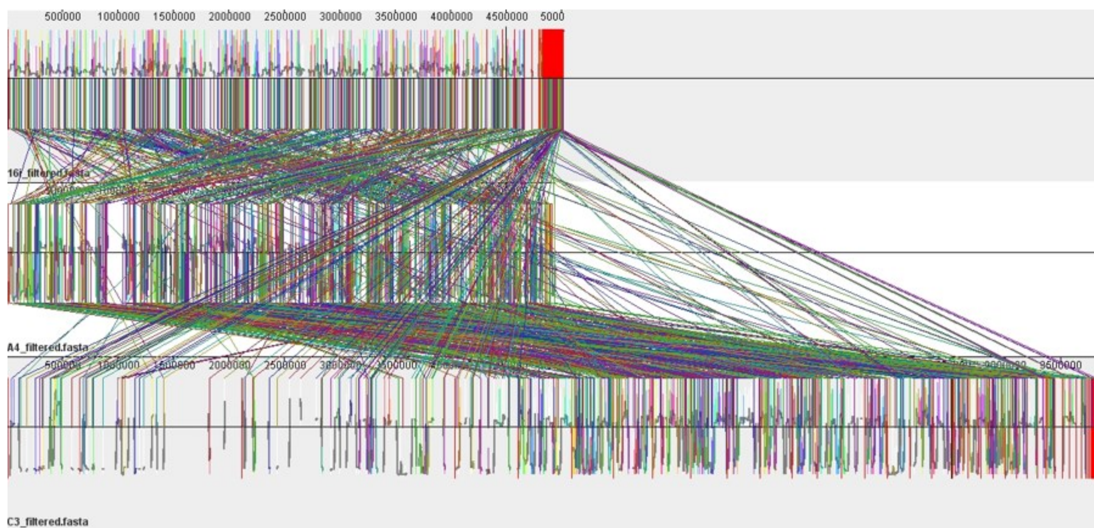

Figure 11 ProgressiveMAUVE alignment of three draft genomes. ProgressiveMAUVE three way alignment of the *Enterobacter cloacae* strain 16i draft genome, the *Pseudomonas stutzeri* strain A4 draft genome, and the *Stenotrophomonas maltophilia* strain C3 draft genomes. When compared to the three way alignment of the reference genomes (Figure 10), these draft genomes have increased genetic similarity that would be considered unusual for their order-level phylogenetic differences**.**

The draft genome for *Pseudomonas stutzeri* strain A4 showed 4.9 Mbases, 4447 coding sequences (CDS), 4491 identified genes, and 47 miscellaneous RNA (miscRNA). The draft genome for *Stenotrophomonas maltophilia* strain C3 showed 9.8 Mbases, 9446 coding sequences, 9620 genes identified, and 147 miscellaneous RNA. The draft genome for *Enterobacter cloacae* strain 16i showed 4.8 Mbases, 4393 coding sequences, 4628 genes identified, 235 miscellaneous RNA, and 2 CRISPR protein related sequences (Figure 12).

```
organism: Genus pseudomonas stutzeri
contigs: 18
bases: 4925180
CDS: 4447
gene: 4494
misc_RNA: 47
```

```
organism: Stenotrophomonas maltophilia strain
contigs: 89
bases: 9794812
CDS: 9446
gene: 9620
misc_RNA: 174
```

```
organism: Enterobacter cloacae strain
contigs: 35
bases: 4755097
CDS: 4393
CRISPR: 2
gene: 4628
misc_RNA: 235
```

Figure 12 Prokaryotic genome annotation output for three draft genomes. Output from PROKKA .log files after the annotation of the three separate draft genomes of the putative PGPR, *Pseudomonas stutzeri* strain A4 **(left),** *Stenotrophomonas maltophilia* strain C3 **(center),** and *Enterobacter cloacae* strain 16i **(right)**, respectively**.**

Discussion

From this study, we were able to conclude that by combining Illumina sequencing short, paired end reads, and ONT MinION long, single-end reads we could produce high-quality draft genomes. The high quality draft genomes were able to be preliminarily annotated with a prokaryotic genome annotation pipeline, producing output that confirmed the quality of the genome assembly, and providing confirmation that the genomes could be run through the NCBI PGAP pipeline for future annotation (outside the scope of this study).

In the bootstrapping methodology implemented, it was clear that we could provide high-quality genome assembly with or without the use of the ONT MinION due

to the relative lack of complexity when assembling shorter prokaryotic genomes. In the case of *Stenotrophomonas maltophilia* strain C3, the addition of the ONT MinION reads at the final round of bootstrapping actually decreased the average mean length of the contigs, which is not desirable in genome assembly. For all three draft genome assemblies, the addition of the ONT MinION reads in the final round of bootstrapping may have decreased the total number of contigs in the assembly, but it also increased the number of contigs that were under 1kb in length, and therefore had to be excluded from further downstream analysis. I do not believe, that in the case of these prokaryotic draft genome assemblies, the addition of ONT MinION reads increased the quality of the draft genomes in the way the manufacturers predict and market.

The results from the preliminary prokaryotic genome annotation show that there were not any pseudogenes present in out draft genomes, meaning that the overall assembly is of high quality. Because we were able to confirm the relative quality of the draft genome assemblies, we plan to continue the annotation of the three draft genomes using NCBI PGAP, and obtain accession numbers for the genomes after submission. Very few high quality rhizobacteria whole genome assemblies and annotations exist, so we are providing a new resource for the PGPR community.

CHAPTER IV

CONCLUSIONS

The encompassing goal of this research was to identify a method of PGPR inoculation (single isolate and consortia) for the alleviation of drought stress in wheat seedlings. We designed our experiment to include root inoculations of putative PGPR, which were naturally occurring rhizobacteria in soil that was subsequently bioprospected from primed, drought-tolerant soil inoculum that had undergone HMME for a drought tolerant aboveground phenotype.

In the first chapter of this study, using the drought tolerant rhizosphere microbiome as the original inoculum for bioprospecting, we were able to isolate and culture 64 putative PGPR that were tested in plant assays for their ability to confer drought tolerance to TAM111 wheat seedlings in a growth chamber environment. Our rapid screening method and study of phenotypic performance narrowed down our search to 12 of the 64 isolates being putative PGPR. These isolates were considered for our downstream consortia assays, due to their ability to confer a drought tolerant phenotype to aboveground plant tissue, when compared to the controls.

From the 12 putative PGPR identified in this study, I chose to use a *Pseudomonas stutzeri* (A4), *Stenotrophomonas maltophilia* (C3), and previously identified PGPR (positive control) *Enterobacter cloacae* (16i) isolate for the consortia studies. These rhizobacteria isolates were chosen based on their ability to colonize

quickly, their individual ability to confer a drought tolerant phenotype in TAM111 wheat seedlings, and their phylogenetic distance from one another.

From the consortia assays we were able to deduce that the individual PGPR inoculations were providing a positive aboveground phenotype when compared to the consortia combinations and the controls. However, the measured belowground phenotype of the root architecture in the treatment groups did not elucidate that enhanced root architecture was the mechanism through which the PGPR strains were providing plant beneficial phenotypes.

Future studies to elucidate the mechanisms in which *Pseudomonas stutzeri* strain A4, *Stenotrophomonas maltophilia* strain C3, and *Enterobacter cloacae* strain 16i confer the aboveground phenotype of drought tolerance would include, but not be limited to the following:

1. LC-MS metabolomics profiling *in planta* of the different plant tissues when inoculated with each individual PGPR under drought stress.
2. Studies on stomatal opening and closing of plants when treated with the individual PGPR under drought stress.
3. Studies examining whether or not ROS scavenging enzymes are present after inoculation of the individual PGPR under drought stress.
4. Assays to characterize exopolysaccharide production in the plant root rhizosphere with the individually inoculated PGPR under drought stress.

Future research would need to include field-level trials of the PGPR treatments to see how they interact with native biota, alongside biofumigation treatments that would

hypothetically “clear the niche” so the PGPR have a greater opportunity to colonize the root rhizosphere without native biota competition or antagonism.

In the second chapter of this study, we were able to used high-quality genomic DNA from our PGPR isolates to sequence, assemble, and annotate high quality whole draft genomes of each PGPR.

Using our hybrid sequencing technique and a unique pipeline to accommodate for the differences in Illumina sequencing output and ONT MinION sequencing output, we were able to assemble the whole genomes of *Pseudomonas stutzeri* strain A4, *Stenotrophomonas maltophilia* strain C3, and *Enterobacter cloacae* strain 16i. In the case of *P. stutzeri* strain A4 and *E. cloacae* strain 16i, the addition of ONT reads seemed to improve the overall assembly. The addition of ONT reads into the assembly of *S. maltophilia* strain C3, showed a decrease in the N50 scores, which is not a favorable outcome of draft genome assembly.

Future studies directly comparing an Illumina sequencing exclusive assembly and our hybrid assembly, side by side, will being to help us elucidate whether or not the long-reads ONT sequencing platforms provided are necessary or even helpful for prokaryotic genome assembly and downstream annotation.

With our preliminary annotation of the *P. stutzeri* strain A4 and *S. maltophilia* strain C3 draft genomes, we plan future studies using the gene predictions provided to discern whether or not the high amount of locally collinear blocks in the MAUVE alignments of the three draft genomes could be a result of horizontal gene transfer or genetic mutations due to their shared priming in a drought tolerant microbiome. Due to

budgetary and time constraints, the further exploration of the gene predictions corresponding to drought tolerant genes identified in different studies will be part of future experimentation and analysis within the scope of this work.

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APPENDIX A

RAPID PLANT SCREENING ASSAY

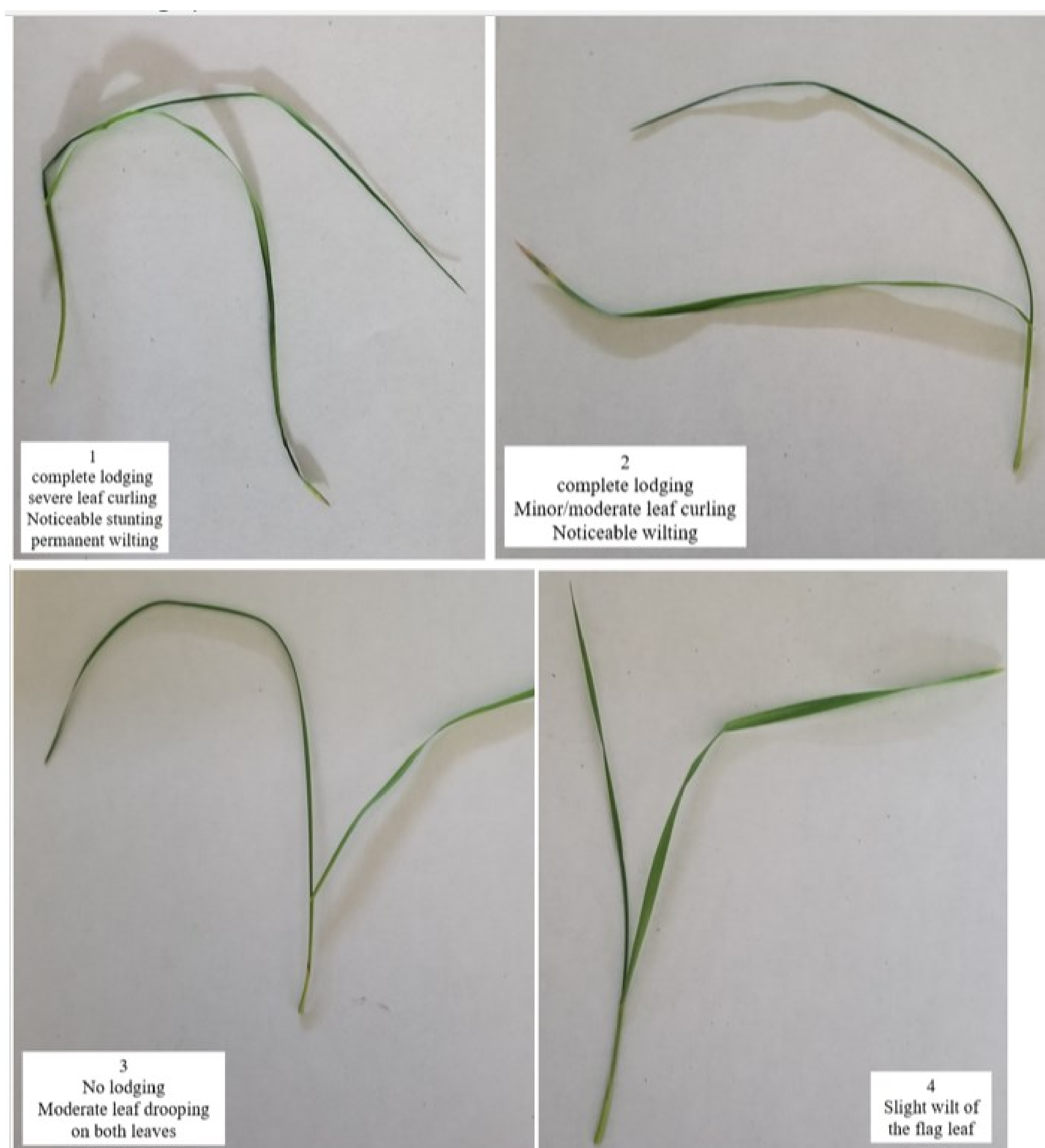

Figure 13 Rapid screen assays for TAM111 wheat seedlings. The rapid screening assay benchmarks were developed by the Jo Lab at Texas A&M University. These benchmarks were used for screening all 64 PGPR isolates and all consortia assays that included all possible consortia combinations. This method allowed us to consistently measure the aboveground phenotype in our wheat seedlings.

APPENDIX B

ABOVEGROUND PHENOTYPE DATA ANALYSIS

Table 7 Qualitative analysis of aboveground plant tissue in consortia assays. Qualitative analysis of aboveground plant tissue in consortia assays (n=100) using the rapid screening assay analyzed using LR Statistics for Type I analysis.

Observation	Treatment	Frequency (n)	Rate (median)
1	A4	100	3
2	C3	100	3
3	16i	100	3
4	A4 + C3	100	2
5	A4 + 16i	100	2
6	C3 +16i	100	2
7	A4 + C3 + 16i	100	2
8	control	100	2

In the consortia assays, qualitative assessment of the aboveground host tissue (Figure 7), via the rapid rating system, showed that single isolate PGPR alleviated drought stress symptoms when compared to all consortia combinations and the control (Figure 1-2). When treated with single isolate PGPR, the qualitative plant performance suggests that wheat seedlings undergo a delay of onset drought symptoms when compared to the consortia and the control treatments.

LR Statistics For Type 1 Analysis

Source	Deviance	DF	Chi-Square	Pr > ChiSq
Intercepts	11411.3281			
trial	11138.1800	9	136.57	<.0001
tmt	9510.2826	7	813.95	<.0001

Contrast Estimate Results

Label	Mean Estimate	Mean Confidence Limits		L'Beta Estimate	Standard Error	Alpha	L'Beta Confidence Limits		Chi-Square	Pr > ChiSq
16i vs control	0.9574	0.9394	0.9703	3.1132	0.1898	0.05	2.7412	3.4852	269.06	<.0001
Exp(16i vs control)				22.4933	4.2691	0.05	15.5061	32.6292		
a4 vs control	0.9521	0.9323	0.9663	2.9897	0.1871	0.05	2.6231	3.3564	255.43	<.0001
Exp(a4 vs control)				19.8804	3.7190	0.05	13.7782	28.6852		
a4_16i vs control	0.6084	0.5164	0.6934	0.4408	0.1915	0.05	0.0655	0.8160	5.30	0.0213
Exp(a4_16i vs control)				1.5539	0.2975	0.05	1.0676	2.2615		
a4_c3 vs control	0.7293	0.6484	0.7974	0.9912	0.1934	0.05	0.6121	1.3704	26.26	<.0001
Exp(a4_c3 vs control)				2.6946	0.5212	0.05	1.8443	3.9368		
a4_c3_16i vs control	0.6636	0.5787	0.7392	0.6796	0.1847	0.05	0.3176	1.0416	13.54	0.0002
Exp(a4_c3_16i vs control)				1.9731	0.3644	0.05	1.3739	2.8337		
c3 vs control	0.9571	0.9389	0.9700	3.1044	0.1898	0.05	2.7325	3.4763	267.65	<.0001
Exp(c3 vs control)				22.2963	4.2309	0.05	15.3713	32.3411		
c3_16i vs control	0.4702	0.3731	0.5695	-0.1195	0.2038	0.05	-0.5190	0.2800	0.34	0.5576
Exp(c3_16i vs control)				0.8873	0.1809	0.05	0.5951	1.3231		
16i vs a4_16i	0.8930	0.8555	0.9217	2.1220	0.1754	0.05	1.7782	2.4657	146.37	<.0001
Exp(16i vs a4_16i)				8.3477	1.4641	0.05	5.9193	11.7722		
a4 vs a4_16i	0.8806	0.8403	0.9118	1.9985	0.1724	0.05	1.6606	2.3364	134.41	<.0001
Exp(a4 vs a4_16i)				7.3780	1.2718	0.05	5.2627	10.3435		
16i vs c3_16i	0.9620	0.9453	0.9738	3.2328	0.1956	0.05	2.8493	3.6162	273.02	<.0001
Exp(16i vs c3_16i)				25.3493	4.9596	0.05	17.2755	37.1966		
c3 vs c3_16i	0.9617	0.9449	0.9735	3.2240	0.1946	0.05	2.8425	3.6054	274.44	<.0001
Exp(c3 vs c3_16i)				25.1273	4.8900	0.05	17.1590	36.7958		
a4 vs a4_c3	0.8806	0.8403	0.9118	1.9985	0.1724	0.05	1.6606	2.3364	134.41	<.0001
Exp(a4 vs a4_c3)				7.3780	1.2718	0.05	5.2627	10.3435		
c3 vs a4_c3	0.8922	0.8544	0.9210	2.1132	0.1753	0.05	1.7697	2.4567	145.38	<.0001
Exp(c3 vs a4_c3)				8.2745	1.4502	0.05	5.8690	11.6661		