GENOMICS OF SOIL ASSOCIATED MICROBIAL COMMUNITIES

by

JOSUE FERNADEZ CANELA

(Under the Direction of Jeffrey L. Bennetzen)

ABSTRACT

The rhizoplane, the surface of plant roots, hosts a distinct microbial community that is crucial for plant-soil interactions (such as mineral uptake). This zone is densely colonized by microorganisms, sometimes forming biofilms that are involved in the early stages of plant root colonization by endosphere microbes. The rhizoplane plays a key role in plant growth and health, as it is the initial site for the establishment of both plant growth-promoting and pathogen-suppressing bacteria. In this study, ten bacterial isolates were obtained by replica plating washed roots from three-week-old maize seedlings. Their genomes were sequenced using Oxford Nanopore long-read technology, enabling chromosome-level assemblies. Comparative genomic analysis revealed significant gene enrichment related to carbohydrate metabolism, motility and chemotaxis, phosphorus metabolism, and sulfur metabolism. Notably, six of these isolates are potentially new species.

To further explore microbe-microbe interactions, a novel technique termed Microbial Partner (MiPner) analysis was developed. This method uses physical binding as a selection

mechanism to identify pairs of interacting bacterial species. A strain of *Serratia marcescens* (SMC43) isolated from soil at the campus of the University of Georgia was selected due to its distinctive red pigmentation, resulting from the production of the antimicrobial compound prodigiosin. This red pigmentation means that MiPner colonies can be seen on a *S. marcescens* background because of contrasting colors. A series of controls were pursued to gain some measure of the frequencies and types of possible false negatives and false positives in the procedure. The MiPner technique was found to dramatically (>100 fold) enrich for members of the genera *Sphingobium* and *Caulobacter*. Results of individual isolates suggest that MiPner technology can isolate one class of bacteria that are otherwise challenging to culture because they require a partner under the plating systems routinely employed to isolate bacteria from any environment.

Additionally, numerous other bacteria were isolated from several soil and/or root environments, and genome sequencing indicated that at least 11 of these are new species. These results show that there is a great deal of bacterial genetic diversity that remains to be discovered in soil and root environments.

INDEX WORDS: soil microbiome, root microbiome, rhizoplane, maize, genome analysis
MiPner, Average Nucleotide Identity, species

GENOMICS OF SOIL ASSOCIATED MICROBIAL COMMUNITIES

by

JOSUE FERNANDEZ CANELA

BS, Autonomous University of Santo Domingo, Dominican Republic, 2018

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2024

GENOMICS OF SOIL ASSOCIATED MICROBIAL COMMUNITIES

by

JOSUE FERNANDEZ CANELA

Major Professor:	Jeffrey L. Bennetzen
Committee:	Katrien M. Devos
	James Lebens-Mack
	Jason Wallace

Electronic Version Approved:

Ron Walcott
Vice Provost for Graduate Education and Dean of the Graduate School
The University of Georgia
December 2024

DEDICATION

To my mother Cristina Canela Gomez, and my sister Ana Cristina Fernandez Canela for their love and support.

ACKNOWLEDGEMENTS

I want to thank my advisor, Dr. Jeffrey Bennetzen, for his invaluable guidance with project ideas, experimental design, data analysis, results interpretation and writing in the past years. I was not able to complete my dissertation without his help.

I want to thank my committee members Dr. Katrien Devos, Dr. James Lebens-Mack, and Dr. Jason Wallace who provided invaluable suggestions on my dissertation in and out of committee meetings.

I want to thank the former research scientist Dr. Srinivasa Chaluvadi who introduced me to microbiome lab work. I want to thank former undergraduate and lab technician Vienna Elmgreen for her help in the field work and starting the MiPner technique. I want to thank Dr. Philip Brailey-Jones for his help with the MiPners. I want to thank my former undergraduate Noelle Svoboda for her help with the work of rhizoplane genomes. I want to thank my last undergraduate Kacee Steinbauer, for helping me finish all my bench work. I want to thank Mike Boyd for his help with greenhouse work. I want to thank Shaugnessy Ryan McCann for his help with the teaching of GENE4240L.

I want to thank my lab mates, alumni and undergraduates, Dr. Cristina Vives Cobo, Dr. Paula Burchardt, Dr. Hongye Zhou, Dr. Shumeng Zhang, Dr. Rocky Giarratano, Ben Long, Olivia Asher, Sailaja Maddali, Ashton Brinkley, Sarah Mondibrown, Aye Htun, Bhavik Sigh, and many more, for academic help, support and fun time together.

I want to thank my friends here in Athens for all their love, support and awesome memories. I want to thank my family who provide unlimited love and support.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

SECTION 1.1

SOIL: HABITAT AND DIVERSITY

Soil is a broad term that generally describes the loose, thin, and variable layer composed of mineral and organic substances, often teeming with biological activity, that blankets much of the Earth's terrestrial surface. This layer plays a crucial role in supporting plant life, regulating water cycles, and serving as a habitat for a diverse array of organisms. Soil formation is influenced by factors such as climate, topography, geological history, and the presence of living organisms, resulting in a wide range of soil types with different properties.

Key structural characteristics of the soil matrix include the formation of clay–organic matter complexes and the stabilization of clay, sand, and silt particles through aggregation (Rabot et al., 2018). These soil aggregates vary in size from about 2 mm or larger (macro aggregates) to microscopic fractions of a micrometer for bacteria and colloidal particles (Daniel 2005). This variability can be seen in aspects such as soil texture, structure, moisture content, and nutrient distribution. These differences can arise due to various influences including the type of parent material, weathering processes, organic matter content, and biological activity. Moreover, soil properties can change over time due to factors like erosion, organic matter decomposition, and human activities such as agriculture and construction. This intricate and dynamic nature of soil plays a crucial role in determining its ability to support plant growth, store and filter water, and sustain diverse ecosystems.

The characteristics of soil are highly variable from location to location and over geologic time. The physical habitat of soil is characterized by physical and temporal heterogeneities across all measured scales, from nm to km (Young and Crawford 2004). The scale of soil particles and the gaps between them can vary dramatically, from as small as 1e-7 m in clays to more than 0.01 m in gravels (Peng, Horn, and Hallett 2015). The interaction of numerous factors results in a large diversity of soil types. While some soil compositions and structures are well-characterized, many are still poorly documented (Rabot et al. 2018). Soil structure plays a key role in influencing numerous soil processes. It affects how water is retained and absorbed, regulates gas exchanges, influences the cycling of organic matter and nutrients, impacts root growth, and is one factor in how vulnerable soil is to erosion (Rabot et al. 2018). Additionally, it provides a habitat for a diverse array of organisms, shaping their diversity and regulating their activity (Rabot et al. 2018). In turn, these organisms modify soil structure, altering the distribution of water, minerals, nutrients and air within their environment (Rabot et al. 2018).

Soil as a microbial habitat

Soil is the most diverse microbial habitat on Earth, holding at least one quarter of its total biodiversity (Sokol et al. 2022; Daniel 2005; Torsvik and Øvreås 2002). There are estimates of a gram of bulk soil can contain 10^4 to 10^7 bacterial cells, and thousands of species. Microbial diversity refers to the complexity and variability across various levels of biological organization (Torsvik and Øvreås 2002). It includes genetic differences within species, the richness and evenness of species and functional groups in communities. At the ecosystem level, important diversity aspects include the variety of processes, interaction complexity, and the number of trophic levels (Torsvik and Øvreås 2002).

Soil microorganisms tend to adhere tightly or absorb onto soil particles like sand grains or clay–organic matter complexes (Daniel 2005; Foster 1988; Paul and Clark 1989). Their microhabitats include the surfaces of soil aggregates and the complex pore spaces both between and within these aggregates (Daniel 2005; Foster 1988). Certain pore spaces are inaccessible to microorganisms due to their small size (Daniel 2005; Peng, Horn, and Hallett 2015). The availability of water and nutrients greatly influences the metabolism and survival of soil microorganisms (Daniel 2005).

Soil microorganisms can be in a state of active growth and foraging for nutrients, but a significant portion also stay dormant due to unfavorable growth conditions (Lennon and Jones 2011; Sokol et al. 2022; Daniel 2005). This dormancy creates banks of microbial diversity within the soil, which can be reactivated when environmental conditions change, such as with the introduction of nutrients from root growth (Lennon and Jones 2011; Kuzyakov and Blagodatskaya 2015). In hotspots, there can be two to 20 times more active bacteria compared to bulk soil (Kuzyakov and Blagodatskaya 2015).

There is no single 'typical' soil microbiome (Fierer 2017). The taxonomic diversity and abundance of individual members in the soil microbiome (community structure) varies widely both across different ecosystems and small-scale habitats (Vos et al. 2013; Fierer 2017; Daniel 2005; Sokol et al. 2022). This variation occurs even when samples are collected just a few centimeters apart (Fierer 2017). Such differences in microbiome composition are partly due to spatial variability in the soil environment and the unique characteristics of each sampling site. However, bacteria and fungi typically dominate the microbial biomass and diversity of the soil (Sokol et al. 2022).

Our understanding of the full extent of soil microbial diversity remains elusive, as there are methodological considerations that can lead to misestimations of diversity: Extracellular DNA from dead microorganisms, which can persist in soil from weeks to years, may increase diversity estimates by an average of 40% (Carini et al. 2016).

The soil microbiome is responsible for essential ecosystem functions and services, such as plant fitness and productivity, nutrient cycling, decomposition of organic matter, pollutant degradation and pathogen control (Delgado-Baquerizo et al. 2020; Fierer 2017).

Microbial growth and survival in the soil environment are often severely constrained (Fierer 2017). Persistent abiotic stressors such as low water availability, limited organic carbon substrates, and acidic conditions pose major challenges (Fierer 2017). Additionally, there is intense competition with other soil microbial taxa, highlighted by the prevalence of antibiotic-producing and antibiotic-resistant soil bacteria (D'Costa et al. 2006; Fierer 2017). Frequent disturbances, including drying-rewetting cycles, predation by earthworms and other fauna, and freezing-thawing events, further hinder microbial community stability (Kuzyakov and Blagodatskaya 2015). Moreover, the uneven distribution of resources across space and time adds another layer of difficulty for microbial communities (Fierer 2017; Kuzyakov and Blagodatskaya 2015). Because of this, despite the high amounts of microbial biomass present in soil, microorganisms typically occupy far less than 1% of the available soil surface area (Young and Crawford 2004). This indicates that biotic or abiotic factors constrain the microbial colonization of soil surfaces (Fierer 2017). Effects of these constrains can be seen when soil is inoculated with large quantities of bacteria, resulting in rapid declines in their abundances (Bashan et al. 2014).

SECTION 1.2

ROOT ASSOCIATED MICROBES

Plants offer numerous niches for the growth and proliferation of diverse microorganisms, including bacteria, fungi, archaea, protists, nematodes, and viruses (Trivedi et al. 2020; Lundberg et al. 2012; Ofek-Lalzar et al. 2014). These are collectively known as the plant microbiota, and the microbes from the plant microbiota can interact with the plant in beneficial, neutral and pathogenic ways (Trivedi et al. 2020; Fitzpatrick et al. 2018). Beneficial interactions can involve processes like nitrogen fixation, nutrient absorption enhancement, and protection against pathogens (Trivedi et al. 2020). Neutral interactions are defined as those where microbes proliferate without significantly affecting the plant. Pathogenic interactions, on the other hand, involve microorganisms that cause diseases and negatively impact plant health. Some microbes may switch between beneficial (symbiotic), neutral and negative (pathogenic) interactions with a plant host, depending on environmental conditions (Johnson, Graham, and Smith 1997; Johnson and Graham 2013). These complex interactions influence plant growth, health, and productivity, highlighting the importance of the plant microbiota in agricultural and natural ecosystems.

The root as a microbial environment

Roots play a vital role in sustaining plant life by absorbing water and essential nutrients from the soil, which are crucial for the nourishment, growth, and development of plants (Ryan et al. 2016). Besides nutrient uptake, roots also offer physical support, anchoring plants firmly in the soil and preventing them from falling over due to wind or other environmental forces (Ryan et al. 2016). This anchoring helps keep plants securely positioned in their habitat, enhancing their overall access to sunlight and, hence, their health and durability (Ryan et al. 2016; Herms et al. 2022). In

addition to these essential roles, roots are a hotspot for microbial diversity (Ryan et al. 2016; Philippot et al. 2013).

Most plant-associated microbes inhabit the root environment, which is divided into three main compartments: the rhizosphere, the rhizoplane, and the endosphere (Figure 1.1) (Edwards et al. 2018; Chaluvadi and Bennetzen 2018; Vandenkoornhuyse et al. 2015). The rhizosphere is the narrow region of soil influenced by root exudates and associated microbial activity but not actually touching the plant (Klein et al. 2022; Philippot et al. 2013). The rhizoplane refers to the root surface, where microbes form close associations with the plant (Schmidt et al. 2018). The endosphere is the internal root tissue, where microbes reside within the plant itself (Xia, Rufty, and Shi 2021; Trivedi et al. 2020). Each of these root-associated compartments exhibits markedly distinct compositional profiles compared to the microbial communities found in unplanted soil (Chaluvadi and Bennetzen 2018; Edwards et al. 2015). This suggests that roots selectively help populate specific subsets of the soil microbiota, fostering unique microbial communities that contribute to plant health and function (Chaluvadi and Bennetzen 2018; Edwards et al. 2015; 2018).

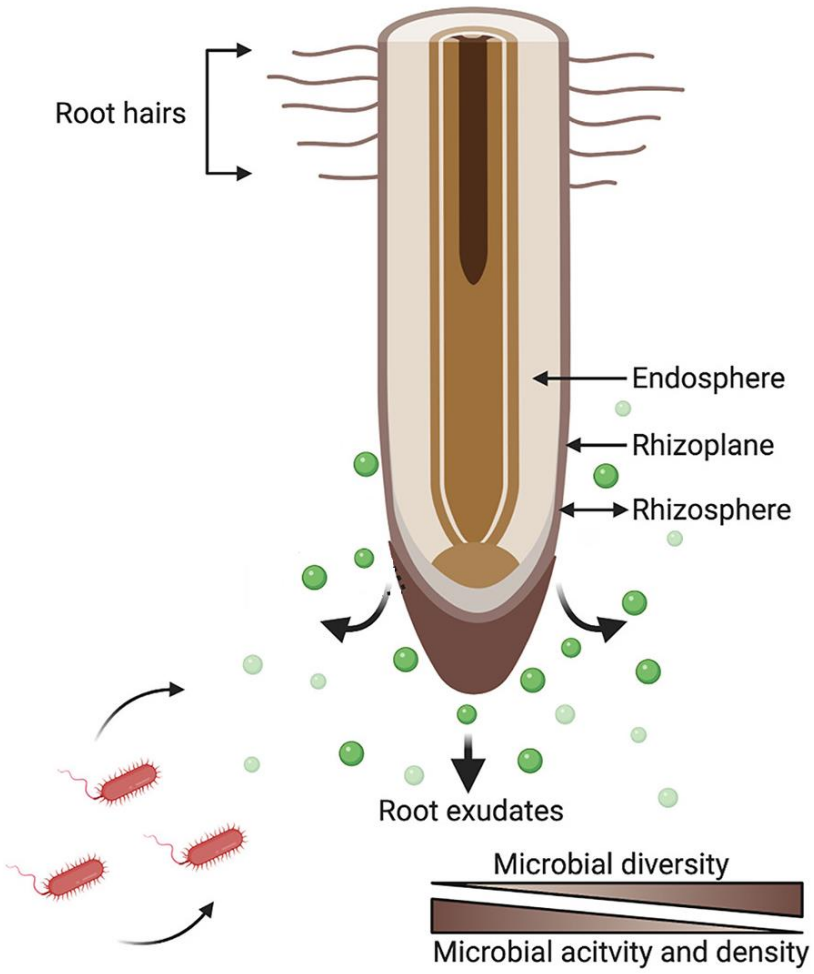

Figure 1.1 Root compartments. Modified from (Knights et al. 2021).

Factors shaping the root microbiome

The majority of root-associated microorganisms are drawn from the soil around them (Vandenkoornhuyse et al. 2015). The root microbiota is heavily influenced by the diversity of microbial species found in the nearby soil (Vandenkoornhuyse et al. 2015). Numerous studies have shown that local environmental factors, particularly soil properties, are crucial in determining the composition of soil microbial communities and root microbiota (Vandenkoornhuyse et al. 2015; Shakya et al. 2013; Schreiter et al. 2014). One key element driving the structure of the root microbiome is the plant itself (Walters et al. 2018; Bulgarelli et al. 2013; Peiffer et al. 2013).

Specifically, plant roots can select and filter soil bacteria, forming unique microbiomes in different sections of the root, such as the rhizosphere, rhizoplane, and endosphere (Chaluvadi and Bennetzen 2018; Edwards et al. 2018). This process is influenced by various root traits, including root exudation and the architecture and morphology of the roots, which impact bacterial colonization and function (Herms et al. 2022).

Root architecture and morphology are essential for plant resource acquisition, they also play roles in shaping the rhizosphere microbiome's assembly and function, but these traits have not been examined as thoroughly as have the roles of root exudation in microbial assembly and function (Herms et al. 2022). Although related, the terms root architecture and root morphology describe two different phenomena. The term root architecture describes the spatial layout of the entire root system, including traits such as the total length, density, branching pattern and intensity, angle, and biomass of the roots (Herms et al. 2022; Bardgett, Mommer, and De Vries 2014). In contrast, root morphology refers to the physical characteristics of individual roots, including diameter, surface area, cell wall composition, presence of root hairs, and specific root length (Herms et al. 2022; Bardgett, Mommer, and De Vries 2014).

Presently, the most extensively studied factor in the development of the rhizosphere microbiome is root exudation (Herms et al. 2022; Sasse, Martinoia, and Northen 2018). Plants exude up to 20% of fixed carbon and 15% of nitrogen (Sasse, Martinoia, and Northen 2018; Haichar et al. 2016). Root exudates, which include low molecular weight molecules like sugars, amino acids, and organic acids, or high molecular weight polymers like mucilage, are secreted by the plant, thereby attracting some bacteria from the soil to the root system (Herms et al. 2022). These exudates create a favorable environment for some microorganisms by serving as a carbon source and providing essential nutrients. This leads to an increase in microbial abundance,

primarily driven by the stimulation of specific microbial populations that thrive on the readily available nutrients. However, this process is accompanied by a decline in microbial diversity. (Ling, Wang, and Kuzyakov 2022; Philippot et al. 2013; Yin et al. 2021). The unique profile of root exudates in a plant significantly influences the recruitment, colonization, and function of specific root-associated bacteria (Herms et al. 2022; Sasse, Martinoia, and Northen 2018). Specific root exudates can even be linked to the attraction of certain bacterial taxa (Neal et al. 2012; Lebeis et al. 2015). The production of bacterial secondary metabolites, such as siderophores, cyclic lipopeptides, and antibiotics, is an important indicator of rhizosphere microbiome functioning and can be influenced by plant root exudates (Herms et al. 2022). Additionally, the secondary metabolites in root exudates can act as antimicrobial agents against pathogens (Olanrewaju et al. 2019). Although every plant produces exudates, variations in root exudates occur across plant species, different growth stages, and among cultivars of the same species (Vieira et al. 2020; Sasse, Martinoia, and Northen 2018; Herz et al. 2018; Aulakh et al. 2001). The chemical composition of root exudates is modified by specific microbial communities through a systemic microbe-to-root signaling mechanism called systemically induced root exudation of metabolites (SIREM) (Kabir and Bennetzen 2024; Knights et al. 2021; Korenblum et al. 2020).

Root compartments

As plants grow, they release exudates that include low and high molecular weight compounds. This process is known as rhizodeposition. The rhizosphere is defined as the narrow zone of soil influenced by root activity. The microbiome of the rhizosphere is different from the bulk soil both qualitatively and quantitatively (Knudsen and Dandurand 2007). Estimates of rhizosphere composition on a single plant are between 10^7 to 10^{12} microbial cells per gram of root and over 30,000 prokaryotic species (Klein et al. 2022; Philippot et al. 2013). Rhizosphere bacteria

experience environmental conditions that differ from those in bulk soil, such as variations in pH, water and oxygen levels, the composition and concentration of growth substrates for bacteria, and the presence of antimicrobial compounds and plant hormones (Vieira et al. 2020). Rhizosphere bacterial communities exhibit higher densities, larger cell sizes, increased microbial activity, and faster turnover rates compared to those in bulk soil (Vieira et al. 2020). While the rhizosphere typically shows reduced microbial diversity compared to bulk soil, it's challenging to provide a consistent description of the rhizosphere microbiome (Philippot et al. 2013). This difficulty arises from significant differences across various studies, which can be attributed to both biological variability and practical issues related to rhizosphere sampling. These issues can be caused by the absence of a distinct physical separation between soil and the rhizosphere which can lead to sampling techniques that do not effectively isolate genuine rhizospheric microorganisms from the bulk of soil microorganisms (Vandenkoornhuyse et al. 2015).

In the rhizosphere, some microbial strains develop particularly close relationships with roots. Some of these microbes attach to the root surface, allowing them to colonize the rhizoplane, and establish themselves as epiphytes (Vandenkoornhuyse et al. 2015). Others penetrate the root and establish themselves in the spaces between cells, known as the apoplast, becoming endophytes (Vandenkoornhuyse et al. 2015). For soil-borne microorganisms, successful establishment in the rhizosphere, colonization of the rhizoplane, and potentially moving inside and within plant tissues are the initial steps toward becoming part of the plant microbiome (Vandenkoornhuyse et al. 2015).

The rhizoplane is the root surface (Schmidt et al. 2018). The rhizoplane contains microorganisms attached to or transiently associated with the root surface, along with many which are embedded in self-produced polysaccharide matrixes known as biofilms (Chaluvadi and Bennetzen 2018; Bogino et al. 2013). The rhizoplane has been proposed as a transient boundary,

where microbes spend part of their life cycle before living as endophytes (Edwards et al. 2018; Vandenkoornhuyse et al. 2015), although it is also likely that some rhizoplane microbes do not have an eventually endophytic fate. The distribution of these microbes in the rhizoplane is affected by the production and release of root exudates, with the root tips and root "cracks" (such as insect damage or the emergence sites of lateral roots) being the main sites for exudation (Pankievicz et al. 2022). Nevertheless, studying the rhizoplane and understanding the process of its colonization is a challenging process due to the technical difficulties of studying the microbes attached to the root surface using culture independent methods (Carroll et al., 2020; Vandenkoornhuyse et al., 2015).

The endosphere is the inside of the root and is colonized by microorganisms that live at least during part of their life cycle inside plant tissue without causing disease (Reinhold-Hurek and Hurek 2011). Among the major sites where roots are colonized by endophytes are intercellular spaces in the epidermal and cortical regions, and inside lysed plant cells. In these locations, endophytes accumulate to high cell densities (Reinhold-Hurek and Hurek 2011). Endophytes can also colonize vascular tissue, including xylem cells, although often in lower densities (Reinhold-Hurek and Hurek, 2011).

The most abundant bacterial groups exhibit similar relative abundance profiles in bulk soil and the rhizosphere, with a slight increase in Proteobacteria in the rhizosphere (Trivedi et al. 2020). However, the bacterial community composition varies significantly between the rhizosphere, rhizoplane and endosphere (Chaluvadi and Bennetzen 2018; Edwards et al. 2015; Trivedi et al. 2020). Endophytic plant communities often show a higher relative abundance of Proteobacteria and Firmicutes (both around twice as high or more compared to the rhizosphere), and somewhat higher levels of Bacteroidetes (Coleman-Derr et al. 2016; De Souza et al. 2016; Fonseca-García et

al. 2016; Hamonts et al. 2018; Trivedi et al. 2020). They are less abundant in Acidobacteria, Planctomycetes, Chloroflexi, and Verrucomicrobia (over twice as low compared to the rhizosphere). Plant-associated archaeal taxa are predominantly classified within the phyla Thaumarchaeota, Crenarchaeota, and Euryarchaeota (Coleman-Derr et al. 2016; De Souza et al. 2016; Fonseca-García et al. 2016; Hamonts et al. 2018; Trivedi et al. 2020). Despite being consistent components of plant-associated communities, the contribution of archaeal communities to host performance remains poorly understood (Trivedi et al. 2020). Recent studies emphasize the considerable diversity and unique niche adaptations of archaeal communities associated with plants, revealing significant differences across various plant parts and showing specific relationships with particular plant species (Trivedi et al. 2020; Taffner et al. 2018; Selim et al. 2022).

Plants have developed symbioses with members of the microbiota since their colonization of land environments over 450 million years ago, and these interactions have significantly shaped plant diversity in terrestrial ecosystems (Delaux and Schornack 2021; Field et al. 2015; Fitzpatrick et al. 2018; Genre et al. 2020). These early associations allowed plants to adapt to terrestrial life by forming mutually beneficial relationships with microbes, such as arbuscular mycorrhizal fungi, which represent one of the most ancient forms of plant intracellular symbiosis (Genre et al. 2020; Strullu-Derrien et al. 2014). Through these symbioses, plants gained enhanced nutrient acquisition capabilities, particularly phosphorus, which was critical for thriving in early terrestrial ecosystems (Delaux and Schornack 2021).

SECTION 1.3

DISCOVERY OF SPECIFIC SOIL ASSOCIATED MICROBES

Initial discoveries

The discovery of soil microorganisms started with the work of Robert Hooke and Antonie van Leeuwenhoek, who used microscopy to observe microorganisms in multiple environments (Gest 2004).

The next achievements in the discovery of soil microorganisms were the advent of pure-culture and staining techniques in the late 19th Century (Escobar-Zepeda, Vera-Ponce De León, and Sanchez-Flores 2015). Pure culture techniques came with the use of nutrient media such as gelatin and potato slides, thus allowing researchers to isolate pure cultures of single microorganisms. These could then be better visualized and physiologically characterized, primarily based on their nutritional requirements. However, this pure-culture approach significantly limited our understanding of microbial diversity, as most microbes cannot be cultivated using standard methods (Pace 1997; Escobar-Zepeda, Vera-Ponce De León, and Sanchez-Flores 2015). Staining techniques, such as Gram, Ziehl–Neelsen, and Schaeffer and Fulton, improved the resolution of microscopy techniques (Escobar-Zepeda, Vera-Ponce De León, and Sanchez-Flores 2015). Microbiologists soon noted a discrepancy: the number of microorganisms observed under a microscope from an environmental sample did not align with those obtained on culture plates derived from those environments (Staley and Konopka, 1985). Although the explanation for this disparity was unclear initially, it was concluded that some microorganisms require unknown conditions for growth. Sergei Winogradsky subsequently devised culture media that mimicked natural environments (McFall-Ngai, 2008).

Sergei Winogradsky is often referred to as the "Father of Soil Microbiology" and made several pioneering contributions to the field (Dworkin and Gutnick 2012). He developed the Winogradsky Column while studying the sulfur cycle and explored microbial growth on CO_2 and inorganic ions, leading to the discovery of chemoautotrophy. Among his key achievements was the study of chemoautotrophic oxidation of ferrous iron and the isolation of *Clostridium*, an anaerobic, spore-forming, nitrogen-fixing bacterium. His work laid the foundation for modern research on anaerobes (Dworkin and Gutnick 2012).

Pioneering work with DNA

The use of molecular sequences to study relatedness between organisms was first proposed by (Zuckerkandl and Pauling 1965). This was followed by the use of DNA-DNA hybridization (DDH) to describe species. This allowed systematists to assay the genomic similarity of two strains, as measured by the fraction of their genomes that are homologous (Cohan 2002). (Johnson 1973) found that strains classified within the same species based on phenotypic characteristics typically shared 70% or more of their genomic content, while strains from different species generally shared less than 70%. As a result, a 70% genomic similarity was established as the gold standard for distinguishing between species (Wayne et al. 1987). DDH has several notable disadvantages: It requires larger amounts of high-quality DNA compared to PCR-based approaches, making the procedure both time-consuming and labor-intensive. Moreover, the various methods available for DDH can produce inconsistent results, especially when dealing with lower reassociation values (Grimont et al., 1980; Huß et al., 1983). The primary drawback, however, is that DDH is a comparative technique, meaning it doesn't support the development of robustly informative databases, unlike sequence information (Gevers et al., 2005; Stackebrandt,

2003). These challenges have led bacterial taxonomists to explore alternative methods that could replace DDH.

A pivotal advance came when Carl Woese used ribosomal RNA squences to study the evolutionary relationships of all organisms, establishing the three domains of life: Bacteria, Archaea and Eukarya (Pace 1997). The application of ribosomal rRNA in microbial studies has significantly accelerated the discovery of new microorganisms compared to traditional methods that relied on morphology and chemical characteristics. In bacterial research, sequence analysis of ribosomal RNA genes has made it easier and faster to determine whether DNA-DNA hybridization is required for species identification. This efficiency stems from the establishment of a threshold that is recognized in species analyzed by both sequence analysis and hybridization. Specifically, if a bacterial strain has less than 97% similarity in its 16S rRNA gene compared to its closest known species, it can be classified as a novel species without the need for further DNA-DNA hybridization testing. This threshold has streamlined the process, enabling faster and more accurate recognition of new bacterial species, significantly accelerating our understanding of microbial diversity (Ogunseitan 2005; Pace 1997).

Metagenomics

Recent advancements in methodology have allowed researchers to fully explore soil microbial diversity and better understand the specific microbial influences on soil processes (Fierer 2017). The use of DNA and RNA based analyses on soil microbiomes has become more common, greatly broadening our comprehension of the phylogenetic and taxonomic structures within soil microbial communities. It is now clear that conventional culture-based techniques significantly and unevenly underestimate the diversity of soil microbes, revealing that soils contain a wide range of microbial taxa from all three domains of life, the great (>90%) majority of which remain

uncharacterized (Fierer 2017; Jansson and Hofmockel 2020; Ramirez et al. 2014; Torsvik and Øvreås 2002).

Currently, the characterization of 16S rRNA gene sequences using next-generation sequencing (NGS) is a standard approach in microbial ecology, allowing for in-depth analysis of microbial communities in soil, the rhizosphere, and the endosphere of plant roots (Viaene et al. 2016; Mendes, Garbeva, and Raaijmakers 2013).

A major advance in the discovery of soil microbes was the development of molecular profiling of communities. Methods include sequencing of microbial marker genes, such as 16s rRNA for bacteria and archaea (Claesson et al. 2010) or the nuclear ribosomal internal transcribed spacer (ITS) region for fungi (Schoch et al. 2012), or shotgun metagenomic sequencing (Bulgarelli et al. 2015; Sharpton 2014).

The use of shotgun metagenomic sequencing has significantly enhanced the precision in identifying microbial community structures beyond the capabilities of 16S rRNA gene amplicon sequencing (Zhu et al. 2022). This enhanced resolution is achieved through the use of algorithms that match high-throughput sequencing-derived DNA sequence data against a comprehensive database of reference genomes, including both whole genomes and marker sequences (Zhu et al. 2022). In a single sequence data set, this technology allows all categories of organisms in the sample to be discovered and quantified, from viruses to archaea to bacteria to eukaryotes of all types. However, the effectiveness of classification depends on the quality and comprehensiveness of the reference database used.

The first large-scale metagenomics sequencing project was conducted by the J. Craig Venter Institute in the Sargasso Sea (Venter et al. 2004). Tringe et al. (2005) did a comparative

metagenomic study comparing marine environmental samples with soil and found that less than 1% of the nearly 150,000 reads generated from the soil library had overlap with reads from other environments. From their 16S rRNA data and genomic sequence overlaps, they calculated that it would take between two and five billion base pairs of sequence to reach the eightfold coverage usually sought for draft genome assemblies, even for the most prevalent genome in this complex soil microbial community (Tringe et al. 2005).

Contemporary technologies

With the decline in cost of whole genome sequencing, the enrichment of databases and increases in computational power, genome-based taxonomy methods have emerged and proliferated (Meier-Kolthoff and Göker 2019). These methods use the entire genome sequence or significant portions of it to determine the relationships between different bacterial species and to classify them accordingly (Hugenholtz, Skarshewski, and Parks 2016; Zong 2020). Genome-based taxonomy methods offer advantages such as a high level of accuracy and resolution in classifying bacteria, and a standardized approach to classification, reducing the subjectivity associated with older methods based on phenotypic characteristics (Hugenholtz, Skarshewski, and Parks 2016; Meier-Kolthoff and Göker 2019; Zong 2020).

New advances in computational power, big data and machine learning have been applied to the discovery of microorganisms (Zha et al. 2022). The application of machine learning techniques has led to the identification of new species from a wide range of taxa (Zha et al. 2022). A notable study recently discovered thousands of previously unknown protistan species on a global scale, demonstrating that protists are distributed in distinct patterns, largely influenced by soil pH (Miao et al. 2020).

SECTION 1.4

MICROBIAL SUCCESSION

Succession

Succession is a basic ecological process, which can be defined as the often orderly and predictable process by which communities change over time following the colonization of a new environment (Fierer 2017). Species community assembly is shaped by two broad types of processes: deterministic and stochastic (Bell et al. 2022; Dini-Andreote et al. 2015). Deterministic processes are governed by abiotic and biotic factors and involve ecological filters, such as homogenizing selection, where communities become more similar than expected by random chance, and heterogeneous selection, where communities become more diverse than expected by random chance (Bell et al. 2022; Dini-Andreote et al. 2015). Stochastic processes include probabilistic dispersal and random fluctuations in species relative abundances (ecological drift) or diversifications that occur independently of environmentally determined fitness (Dini-Andreote et al. 2015; Dini-Andreote and Raaijmakers 2018; Bell et al. 2022). Dispersal refers to the movement of species from one habitat to another, and drift is the random division, death, ecological drift (random fluctuations in species abundance), or diversification (mutation) of individuals within a community (Bell et al. 2022). High dispersal rates across habitats promote homogenization, resulting in similar communities. In contrast, limited dispersal can cause high rates of community turnover, leading to greater differences between communities (Bell et al. 2022; Dini-Andreote and Raaijmakers 2018). The impact of stochastic and deterministic processes on community dynamics changes over space and time (Bell et al. 2022). Factors such as productivity and resource availability play a role in determining the relative significance of these processes.

Succession in microorganisms

A key objective in microbial community ecology is to comprehend the processes driving the observed patterns in species abundances over space and time.

Plants have traditionally been the focus of succession studies due to their perceived physical and ecological dominance in their respective landscapes. Methodological limitations have long hindered ecologists from adequately documenting microbial succession (Fierer et al. 2010). Microbial communities are highly diverse, their composition can change rapidly, and most microbial taxa cannot be identified using standard culture-based methods (Fierer et al. 2010). However, recent advancements in genome sequencing and computational analyses, combined with the enrichment of microbial databases, now allow for comprehensive surveys of microbial diversity and succession patterns with unprecedented ease (Fierer et al. 2010). These methodological improvements align with a growing recognition that studying microbial succession offers unique opportunities for ecologists to test and expand existing conceptual models of ecological succession. Furthermore, research on the changes in abundance of specific microbial taxa during succession may provide crucial insights—potentially the only insights—into the natural history and physiology of the many taxa that resist cultivation and isolation in laboratory settings (Fierer et al. 2010). In a review of microbial succession, Fierer et al. (2010) classified microbial succession into categories based on their carbon source, in autotrophic and heterotrophic succession. In autotrophic succession, the first colonizers are mainly autotrophs that harness energy through light or by oxidizing inorganic compounds. Initially, there is minimal or no organic carbon (C) available, but its supply gradually increases over time. The heterotrophic succession was further divided into exogenous and endogenous categories. Exogenous succession is driven

by continuous external inputs of organic C, whereas endogenous succession relies primarily on a single initial input of organic C contained within the substrate itself (Fierer et al. 2010).

Microbial community assembly has historically been examined from a deterministic angle, with studies showing that a range of environmental factors—like pH, salinity, and organic carbon—affect community formation at various scales (Fierer 2017; Nishida, Nakagawa, and Yamamura 2021). Nonetheless, recent findings have highlighted a growing recognition of the importance of stochasticity in some microbial systems (Bell et al. 2022; Dini-Andreote et al. 2015). Instead of framing the issue as a dichotomy where one rejects stochastic processes in favor of deterministic ones (or the other way around), a broader perspective should be adopted that incorporates both processes and aims to understand how and why their relative impacts differ across systems, temporal scales, and spatial contexts (Dini-Andreote et al. 2015).

As crop plants grow and modify their environment, the impact of deterministic processes is expected to intensify (Bell et al. 2022; Dini-Andreote et al. 2015). This is due to selective pressures that filter the initial microbial community, favoring those microorganisms that are best adapted to the changing conditions. If there is a strong link between microbial communities and improved crop performance, crop development programs could strategically leverage the microbiome at specific phenological stages to enhance plant growth and yield (Bell et al. 2022).

Succession in the roots

Understanding the process of succession community assembly in roots is challenging. Root microbiome assembly is a multistep process influenced by both soil type and host differences (Fitzpatrick et al. 2018). However, our understanding of how variations among and within host species shape rhizoplane and endosphere assembly remains limited (Fitzpatrick et al. 2018). This

understanding is crucial for comprehending how root microbiota contribute to the ecology and performance of their hosts (Fitzpatrick et al. 2018). The formation of microbial communities in the roots from the time a root tip penetrates the bulk soil until the root tissues reach maturity is poorly understood (Dupuy and Silk 2016).

Initial community establishment is expected to be mainly driven by stochastic processes (Dini-Andreote et al. 2015). It has been proposed that the sugars released by seedling roots in soil create a resource-rich environment that reduces competitive pressures, leading to stochastic dominance during the initial establishment of rhizosphere communities (Dini-Andreote et al. 2015). After the initial establishment of microbial communities, deterministic selection can become more influential as organisms modify their surroundings (e.g., by depleting resources) (Dini-Andreote et al. 2015). Therefore, as selection becomes stronger, a wider range of taxa are excluded. Antimicrobial production by initial colonizers is likely to also narrow the diversity of microbial species contributions to community development.

In theory, community assembly starts with the random, unregulated colonization of taxa from nearby bulk soil (neutral processes), a process that continues throughout root lifecycles (Bonkowski et al. 2021). Conversely, structured dynamics (microbiome assembly) occur through selection (niche-based processes) when (i) exudates stimulate the growth of fast-growing copiotrophic taxa, (ii) root signals attract specific symbionts or pathogens, (iii) increased competition due to limited resource availability leads to species sorting, and (iv) predation favors specific microbial traits within the microbiome (Bonkowski et al. 2021). Microbe-microbe competition and collaboration should also contribute to the complexity of this dynamic.

A mathematical model of bacterial growth dynamics following the encounter of a growing root tip by Dupuy and Silk (2016) highlighted early attachment to root tips as a critical bacterial

trait. This is because bacteria that attach early and stay on the maturing root have access to larger amounts of exudate-C, allowing them to multiply more rapidly. Given that bacterial colonization is restricted by a limited number of attachment sites, Dupuy and Silk (2016) proposed that detachment and re-colonization of less populated root tip areas can significantly enhance the microbial carrying capacity of roots, resulting in a pronounced bacterial density peak at the tips.

Research in rice has shown that the root microbiome was highly dynamic during the vegetative phase of plant growth and then stabilized compositionally for the remainder of the life cycle (Edwards et al. 2018; 2015). They found that rice seedlings acquire root-associated microbiomes quickly, with microbial penetrance into the endosphere occurring within 24 hours of transplantation into field soil (Edwards et al. 2015). Microbiome acquisition from soil appears to involve a multistep process, where many microbes first colonize the rhizosphere, then the rhizoplane, and eventually migrate to the root's interior (Edwards et al. 2015).

In a study examining microbial succession in maize under four different management conditions, Bourceret et al. (2022) identified 26 stable bacterial operational taxonomic units (OTUs) in the root compartment. These OTUs included 16 Proteobacteria and 10 Actinobacteria, collectively accounting for over half (50.94%) of the root microbial community in terms of aggregated relative abundance (aRA). Notably, 15 of these OTUs, predominantly Proteobacteria, were also present in both the root and rhizosphere compartments. In the rhizosphere, these shared OTUs contributed 17.30% and 20.89% to the aRA during the vegetative and reproductive growth stages, respectively, with their abundance increasing significantly in the root (to 37.44% and 39.35%, respectively), regardless of field location or soil management. This suggests a progressive enrichment of these OTUs as they transition from the rhizosphere to the root. A similar enrichment pattern was observed in the fungal community (Bourceret et al. 2022).

Research in canola (*Brassica napus*) found that microbial succession in roots was dominated by stochastic community assembly processes, while the rhizosphere was a highly selective environment (Bell et al. 2022). The assembly processes in all plant compartments were primarily influenced by the plant's growth stage, with minimal impact from different plant lines. In the root endosphere, stochastic effects were likely driven by factors such as competitive exclusion or priority effects.

Succession in roots can be affected by drought (Xu et al. 2018). In sorghum (*Sorhum bicolor*), preflowering and postflowering drought treatments led to a reduction in microbial diversity within the root (by ~20%) and rhizosphere (by ~15%) compared to control plants (Xu et al. 2018). They found that microbial diversity in the rhizosphere and root increased rapidly after seedling emergence (weeks 1-2) and decreased slightly a week later (week 3).

SECTION 1.5

SPECIES CONCEPTS IN BACTERIA

Two key tools for organizing biological diversity are taxonomy and phylogeny (Martiny et al. 2015; Washburne et al. 2018). Taxonomy categorizes microorganisms within a hierarchical structure, from the three domains (Bacteria, Archaea, and Eukarya) down to millions of species (Washburne et al. 2018). Phylogeny, on the other hand, estimates the evolutionary history of microorganisms, classifying each organism based on a series of splits that represent speciation events from a common ancestor into two distinct species (Hug et al. 2016; Martiny et al. 2015; Washburne et al. 2018).

Although microbial taxonomy and phylogeny may eventually align, with every clade in the phylogeny having a corresponding taxonomic name, current taxonomy is relatively coarse

(Washburne et al. 2018). Modern taxonomic labels represent only a small portion of the branches in phylogeny. For now, phylogeny provides a more detailed framework for classifying microorganisms (Hug et al. 2016; Martiny et al. 2015; Washburne et al. 2018).

The concept of biological diversity assumes consensus on the distinct nature of individual species and the mechanisms driving speciation (Ogunseitan 2005). However, recognizing and understanding differences and similarities among microorganisms is more challenging and less well understood compared to large multicellular organisms (Cohan 2002; Ogunseitan 2005). For half a century, bacteriologists have independently grappled with the concept of species, largely disregarding input from nonmicrobial biologists and philosophers (Cohan 2002; Bapteste et al. 2009). Their main challenge has been linking broad prokaryotic species concepts—which involve theories about genetic and ecological processes leading to distinct groups of phenotypically and genomically similar individuals—with workable species definitions that can be used to identify these groups in the lab or nature(Cohan 2002; Ogunseitan 2005).

It has been argued that bacteria should not be grouped into species but rather viewed as a continuum of organisms with varying degrees of divergence based on their evolutionary history (Bobay 2020; Bapteste et al. 2009; Doolittle and Zhaxybayeva 2009). However, microbiologists typically recognize and label bacterial isolates by observing their unique phenotypic characteristics, and genomic analyses show that bacteria form clear clusters of closely related individuals, not a random spread (Bobay 2020; Riley and Lizotte-Waniewski 2009; Caro-Quintero and Konstantinidis 2012; Konstantinidis, Rosselló-Móra, and Amann 2017). This indicates that bacteria can be organized into species. Additionally, bacteria can be classified ecologically based on their common niches and properties (Bobay 2020).

The beginnings of bacterial systematics paralleled those of animal and plant systematics. In the absence of a broadly accepted species theory, systematists identified species as phenotypic clusters (Cohan 2002). While macrobiologists primarily assessed morphological traits and microbiologists focused on metabolic traits, systematists from all major groups managed to delineate biological diversity into phenotypic clusters, which they recognized as species (Cohan 2002). With the development of the biological species concept, the fields of macrobial and microbial systematics diverged (Cohan 2002).

There are four major species concepts that can be compared and contrasted: the typological species concept, the morphological species concept, the biological species concept, and the evolutionary species concept (Ogunseitan 2005).

The typological species concept predates evolutionary theory and defines species based on a "type specimen," reflecting Platonic and Aristotelian ideas of an ideal archetype (Ogunseitan 2005). This concept sees species as static entities with uniform morphological characteristics, representing imperfect manifestations of an eternal, perfect form (Ogunseitan 2005). However, this view has been challenged by modern understanding of variation within populations, where individuals of the same species can differ significantly (Ogunseitan 2005).

The morphological species concept defines species based on anatomical characteristics, making it useful for classifying large groups like fossils and organisms that do not reproduce sexually (Ogunseitan 2005). However, its reliance on expert interpretation of morphological differences can lead to subjective classifications. It fails to explain sympatric species that look identical but are reproductively isolated, nor can it distinguish cryptomorphic species with genetic differences but no morphological changes. One example of the limitations of this method when working with bacteria is that in soils, numerous bacillary bacteria present similar colony

morphologies when grown in culture, but molecular analysis reveals significant physiological and genetic differences among them (Ogunseitan 2005).

The biological species concept was developed by Ernst Mayr and defines species as populations capable of interbreeding that are reproductively isolated from other groups. This concept emphasizes species as ecological and genetic units, where genetic exchange mechanisms constantly shape the population gene pool. While effective for many organisms, including eukaryotes, the biological species concept does not fully apply to prokaryotes. The ability of microbial communities to undergo natural transformation (absorbing genetic material from their environment, including other microbial "species") makes species concepts relying on permanent reproductive isolation inadequate for prokaryotes (Ogunseitan 2005).

The evolutionary species concept was proposed by paleontologist George Gaylord Simpson, to address the inadequacy of the biological species concept when dealing with organisms that reproduce asexually (Ogunseitan 2005). This defines a species as a lineage of populations maintaining its identity and evolutionary tendencies over time (Ogunseitan 2005). This concept is particularly useful in analyzing fossil records. The evolutionary species concept has evolved into various forms, such as the phylogenetic species concept, which defines species as monophyletic groups descended from a single ancestor. However, this concept struggles with genomic hybrids, where genes have been exchanged between taxa. Studies of microbial genomes show that 5% to 15% of the average bacterial genome consists of genes acquired from other "species", making the evolutionary species concept less applicable to prokaryotes (Ochman, Lawrence, and Groisman 2000; Ogunseitan 2005).

The conceptualization of microbial species has often mirrored the criteria used for eukaryotic species (Cohan 2002; Ogunseitan 2005). (Ravin 1960) sought to adapt the biological

species concept for bacteria by defining "taxospecies," which are phenotypic clusters identified through numerical taxonomy, representing groups of organisms with high phenotypic similarity. This is akin to the morphological species concept but includes physiological features. Ravin, (1960) also proposed "genospecies," groups of bacteria capable of gene exchange, but found little correlation between taxospecies and genospecies.

Bacterial species are typically defined using a combination of criteria, as proposed by (Vandamme et al. 1996). The most influential criterion, proposed by Wayne et al. (1987), considers a bacterial species as a collection of strains that share at least one diagnostic phenotypic trait and exhibit at least 70% DNA–DNA hybridization (DDH) (Konstantinidis, Ramette, and Tiedje 2006). In DDH, the measurement evaluates how effectively DNA molecules hybridize rather than directly quantifying sequence identity. Consequently, reaching 70% DDH does not signify 70% sequence identity (Konstantinidis, Ramette, and Tiedje 2006).

Using FastANI, Jain et al., (2018) computed pairwise average nucleotide identity (ANI) values for all prokaryotic genomes in the NCBI database. They found a clear genetic discontinuity: 99.8% of the 8 billion genome pairs analyzed showed >95% ANI within species and <83% ANI between species. This discontinuity holds true even without the most frequently sequenced species and remains consistent over time.

SECTION 1.6

BACTERIAL BINDING

Bacterial binding to surfaces

Bacteria can adhere to a wide range of surfaces, including glass, metals, various polymers, other bacteria, and eukaryotic cells (Kimkes and Heinemann 2020; Tuson and Weibel 2013). In

fact, it is virtually impossible to create a surface that is resistant to bacterial colonization and biofouling while also being safe for humans and the environment (Kimkes and Heinemann 2020).

The understanding of the interaction of bacteria with surfaces is important due to its implications in areas such as bioenergy, biofouling, biofilm formation, and the health of plants and animals (Kimkes and Heinemann 2020). Bacterial attachment to surfaces has been a field of research since the second half of the 20th century (Kimkes and Heinemann 2020).

Little is known about how bacteria initially sense the surface, which leads to adjustments from the planktonic state to a sessile (surface attached) state. Adhering to surfaces offers numerous benefits for bacteria. When bacteria attach to horizontal surfaces, their growth is stimulated, especially in environments with limited nutrients. This is because organic material in the liquid settles and accumulates on these surfaces, increasing the local nutrient concentration (Tuson and Weibel 2013). Similarly, increasing the surface area of the substrate, such as by adding glass beads to a culture container, creates more areas for nutrients to adsorb. This allows bacterial cells to thrive even at nutrient concentrations that would typically be too low to support growth (Tuson and Weibel 2013).

Besides aiding in nutrient capture, surface attachment allows some bacteria to directly obtain essential metabolites and co-factors from the surfaces they adhere to. For instance, bacteria such as *Shewanella* and other related genera that thrive on metal surfaces can utilize metals like iron and magnesium as terminal electron acceptors during respiration (Grant et al. 2023; Nealson and Finkel 2011; Tuson and Weibel 2013).

Bacterial attachment to surfaces is often followed by the formation of biofilms. Biofilms are microbial communities, either from a single species or a consortium of multiple species, where

cells are firmly attached to a surface and embedded in a matrix of extracellular polymeric compounds (Bogino et al. 2013; Fujishige et al. 2006; Ramey et al. 2004). These mixed biofilm compounds (matrices) routinely include exopolysaccharides, proteins, and DNA. The extracellular polymeric substance secreted by cells within these biofilms protects them from mechanical damage and shear forces caused by fluid flow (Tuson and Weibel 2013), and from many other stresses such as desiccation and predation (Bogino et al. 2013).

Binding to surfaces also has several disadvantages, including the inhibition of motility (Tuson and Weibel 2013). This often is associated with a "switch" in gene activation: the same transcriptional regulator that activates genes for extracellular matrix production may deactivate genes for flagella production (Blair et al. 2008; Krasteva et al. 2010; Tuson and Weibel 2013). Inhibiting cell motility prevents bacteria from seeking optimal environments when nutrients are scarce. However, in certain environments, bacterial cells can overcome this disadvantage by sensing surfaces and triggering surface-associated phenotypes that promote motility and prevent adhesion. For example, swarming allows cells to remain motile while capturing nutrients, providing similar advantages to surface adhesion. Additionally, some pathogens use surface sensing to upregulate virulence factors in preparation for host invasion (Tuson and Weibel 2013).

Bacterial binding to root surfaces

Bacterial attachment to roots has been widely studied in agriculturally important bacteria such as the Gram negatives *Rhizobium*, *Agrobacterium*, *Pseudomonas*, *Azospirillum* and *Salmonella*, (Rodri-guez-Navarro, Dardanelli, and Ruiz-Sainz 2007; Wheatley and Poole 2018) and Gram positives such as *Bacillus* and *Streptomyces* (Beauregard et al. 2013; Viaene et al. 2016).

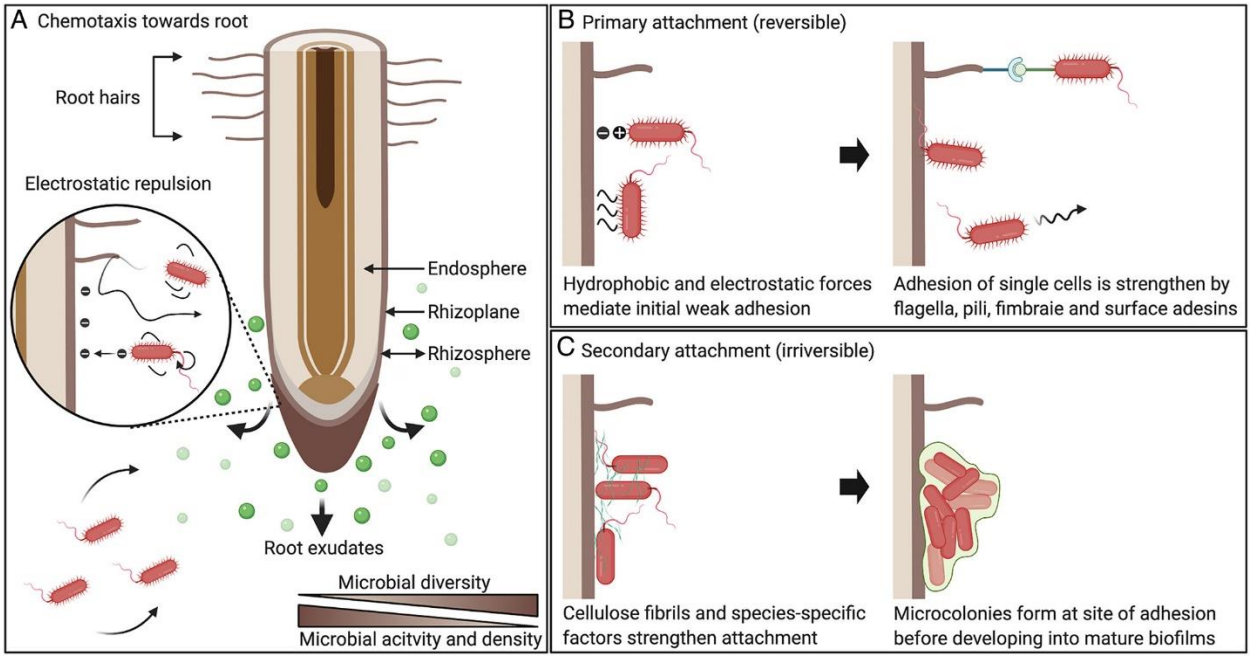

Figure 1.2 The attachment and colonization of plant roots by bacteria is a complex, multistep process. A. Plants release photosynthetically derived carbon compounds and perhaps other signals into the rhizosphere, creating chemical gradients that attract motile bacteria from the soil toward the root surface through chemotaxis. Bacterial movement, driven by flagella and pili, helps them overcome any electrostatic forces that might repel them from the root. **B.** In the initial attachment phase, bacteria weakly and reversibly bind to the root surface through hydrophobic and electrostatic interactions, which are later reinforced by protein appendages and surface adhesins unique to each species. **C.** In the secondary attachment stage, bacteria become strongly and irreversibly attached to the root, leading to microcolony formation. This is facilitated by the production of cellulose fibrils and other species-specific molecules like extracellular polysaccharides and proteins (e.g., biofilms). Figure from (Knights et al. 2021).

Bacteria bind to root surfaces in a biphasic process (Figure 1.2). The initial phase involves a weak, reversible, and non-specific binding of bacteria to the root surface. This binding is driven by hydrophobic and electrostatic forces between bacterial cells and surface molecules on the root,

facilitating initial attachment (Knights et al. 2021; Rodriguez-Navarro et al. 2007; Wheatley & Poole 2018). Despite the advantages of root attachment, only a small percentage of inoculated isogenic bacteria, usually ranging from 0.4% to 3.5%, successfully attach to roots in controlled environments (Rodríguez-Navarro et al., 2007). This low attachment rate is primarily due to electrostatic repulsion between the negatively charged bacterial envelope and the root surface (Knights et al. 2021; Berne et al. 2015). Bacteria overcome this repulsion by using flagella and pili to propel themselves toward the root. After initial contact, cell surface adhesins facilitate a stronger, but still reversible, attachment to the root (Knights et al. 2021; Wheatley and Poole 2018). Primary bacterial adhesins involved in this process include proteinaceous structures such as flagella, pili, fimbriae, as well as surface proteins, exo- and capsular polysaccharides (Knights et al. 2021). Numerous studies have demonstrated the role of flagella and pili as adhesins, enabling bacteria to not only move to the root but also to attach and migrate across the root surface (Knights et al. 2021).

Since bacterial mutants that lack flagella, pili, and fimbriae are still capable of adhering to root surfaces (Knights et al. 2021; Tan et al. 2016), it is likely that other species-specific factors with adhesive properties, like polysaccharides and surface proteins, are important in the initial attachment process. For example, in *Azospirillum brasilense* and *Pseudomonas fluorescens*, certain major outer membrane proteins (MOMPs) have been linked to root adhesion and cellular aggregation (Knights et al. 2021; Mot and Vanderleyden 1991; Burdman et al. 2001; Alvarez Crespo and Valverde 2009). These MOMPs are present on the outer bacterial membrane and function by interacting with surface proteins and polysaccharides on the root's exterior.

This primary attachment is then followed by a secondary phase, where the bacteria shift to a stronger, more specific binding mode, mediated by the synthesis of extracellular cellulose fibrils

and species-specific secondary attachment factors (Rodriguez-Navarro, Dardanelli, and Ruiz-Sainz 2007; Wheatley and Poole 2018; Knights et al. 2021). Biosynthesis, secretion or exposure of these cellulose fibrils and secondary attachment factors is typically induced after successful primary attachment (Knights et al. 2021). Secondary attachment leads to the development of a bacterial microcolony on the root surface, which anchors the bacteria to the rhizoplane. This attachment is essential for many bacteria to proceed with endophytic colonization (Knights et al. 2021).

Bacteria using this biphasic root attachment method participate in a wide array of plant interactions, including roles as plant-growth enhancers, biofertilizers, and pathogens. A common factor in these plant–microbe interactions is the initial attachment to the plant roots. In some cases, the bacteria stay on the root's outer surface, whereas in others, attachment is essential for endophytic or pathogenic colonization inside the root (Wheatley and Poole 2018).

CHAPTER 2

GENOMIC ANALYSIS OF TEN BACTERIAL ISOLATES FROM THE MAIZE RHIZOPLANE

Introduction

Recent advances in genome sequencing and bioinformatics have greatly enhanced our understanding of plant-associated bacteria. The adoption of cultivation-independent methods, such as marker gene profiling and shotgun metagenome sequencing, has significantly expanded our knowledge of microbial ecology in plant environments (Levy et al. 2018). Despite the advantages of current metagenomic methods, isolated genomes are still useful to offer genomic and evolutionary context for individual genes. Isolated rare organisms, and hence their genomes, might be overlooked by metagenomics due to limited sequencing depths (Levy et al. 2018).

Like other eukaryotes, plants and their microbiomes function as holobionts, with plants depending on soil microbiota for certain functions and traits (Mendes et al. 2011). In exchange, plants release up to 21% of their photosynthetically fixed carbon into the rhizosphere, nourishing microbial communities and affecting their activity and diversity (Mendes et al. 2011).

Root-associated microbiomes play crucial roles in enhancing plant nutrition (Trivedi et al. 2020). Some key molecular mechanisms for nutrient acquisition are well understood in symbiotic relationships with arbuscular mycorrhizal fungi (AMF) and *Rhizobium* bacteria (Trivedi et al. 2020). Non-symbiotic plant-growth-promoting bacteria can also increase nutrient bioavailability and improve root system architecture, aiding in resource exploitation. Root-associated

microbiomes also help plants access nutrients like inorganic phosphate and iron through solubilization, mineralization, or uptake via siderophores (Trivedi et al. 2020).

Plant-associated microorganisms can influence plant evolutionary responses to environmental stress in three main ways: by altering the fitness of individual plant genotypes, the expression of plant traits related to fitness, and the strength or direction of natural selection occurring within populations that experience environmental stress through the microorganisms' effects on reproductive fitness (Trivedi et al. 2020).

The rhizoplane is the root surface. The rhizoplane community is taxonomically distinct from the broader rhizosphere (Chaluvadi and Bennetzen 2018). It is colonized by microorganisms that are firmly attached to the root surface, many of which are embedded in a matrix of self-produced polysaccharides and, often, other polymers known as biofilms. This is a critical area of needed study into plant microbe interactions, partly because the colonization of the rhizoplane is a crucial step before the internalization and translocation of endophytic bacteria within plant tissue (Knights et al. 2021; Vandenkoornhuyse et al. 2015). The root surface is where many plant growth-promoting and pathogen-suppressing bacteria establish themselves and exert their influence on the plant. In this zone the bacteria have close access to root exudates, and the plant has close access to bacterial metabolites (Schmidt et al. 2018). Although rhizoplane colonization offers clear benefits, generally only a small percentage (0.4 – 3.5%) of bacterial populations are known/predicted to form attachments with roots, though this number may be a huge underestimate (Rodríguez-Navarro et al. 2007).

Functional traits of microbes can indicate how they thrive, and particularly how they respond and adapt to variations in resources and stress (Pühler et al. 2004). In order for bacteria to live in host-associated environments, such as the rhizoplane, they presumably require the genetic

potential to use host nutrients, avoid or suppress host immunity and compete with other bacteria for the niches in the rhizoplane (Klein et al. 2022; Yu et al. 2019).

A large-scale analysis of plant-associated bacterial isolates found increased carbohydrate metabolism and fewer mobile elements (Levy et al. 2018). Bacteria living in the rhizoplane are likely to require genes involved in processes such as motility and biofilm formation (Knights et al. 2021; Trivedi et al. 2020). Research has consistently shown that when bacterial pathways for chemotaxis or motility are inactivated, there is a significant reduction in rhizoplane colonization (Allard-Massicotte et al. 2016; De Weert et al. 2002; Knights et al. 2021). Understanding the unique features and gene functions of rhizoplane association may offer valuable insights for harnessing beneficial plant-microbe interactions. Such insights will contribute to a broader understanding of root-associated microbial communities and their potential applications in improving plant health and agricultural sustainability.

Methods

Sample collection and isolation

Seeds of maize (*Zea mays*) B73 were surface sterilized by placing them in a 10% bleach solution for 30 minutes, after which they were washed with sterile water 3 times, soaked overnight in 1% Captan (a fungicide), and transferred to pots with soil from a field plot near the Plant biology greenhouses on the campus of the University of Georgia (33° 55’ 44.9’’ N, 83° 21’ 46.1’’ W). After three weeks, the seedlings were collected and moderately washed with 0.1X saline-sodium citrate (SSC) buffer. The roots were carefully placed on 150 mm x 15 mm Petri dishes, containing 0.1X Difco Nutrient Agar media (Figure 1). The Petri dishes were incubated at room temperature (approximately 22 °C) for 5 days. Apparent single colonies were picked and serially streaked onto

the same media three times, and then individual colonies were picked into nutrient broth for growth and eventual storage at -80°C in 25% glycerol.

DNA extraction and whole genome sequencing

High molecular weight DNA was extracted from liquid cultures using the protocol of (Mayjonade et al. 2016). DNA libraries were prepared for sequencing using the Rapid Barcoding Kit following the manufacturer's instructions (SQK-RBK004, Oxford Nanopore Technologies, Oxford UK). The libraries were sequenced for 72 hours using a R9.4.1 flow cell (FLO-MIN106, Oxford Nanopore Technologies) on the GridION instrument.

Overall pipeline of computational analyses

Raw reads were corrected and then assembled using Canu (Koren et al. 2017) using the default settings. The assemblies were circularized using Circlator (Hunt et al. 2015) using the default settings. The circular assemblies were taxonomically identified using Type (Strain) Genome Server (TYGS) (Meier-Kolthoff and Göker 2019). The assemblies were visualized in BRIG (Alikhan et al. 2011), implemented in Proksee (Grant et al. 2023). The genomes were annotated using RAST (Aziz et al. 2008) and Prokka (Seemann 2014). The assembled chromosomes were blasted to the NCBI (RefSeq) database (Pruitt 2004), the five most closely related genomes were downloaded and analyzed with OrthoANI (Lee et al. 2016). The most-closely-related genome to each purified rhizoplane bacterium, that had been sequenced, was identified by OrthoANI (Lee et al. 2016), and annotated using Prokka (Seemann 2014). The gene contents between each rhizoplane genome and the most closely related genome were compared using Roary using a core gene alignment (Page et al. 2015).

RAST annotations of the ten genomes and their closest relatives were manually inspected for genes involved in the following subsystems: Carbohydrates, Motility and Chemotaxis, Iron Acquisition and Metabolism, Phosphorus Metabolism, Sulfur Metabolism, and Nitrogen Metabolism. An R script was used to visualize gene content in these categories and conduct t-tests to identify statistically significant differences.

Results

Isolation of bacteria

Bacteria formed colonies where the root was placed (Figure 2.1). Since the root has a three-dimensional structure, it is difficult to get a fully perfect print of the root as they are pressed against the plate (Figure 2.2). Ten samples with the best DNA yield were sequenced using Oxford Nanopore technologies.

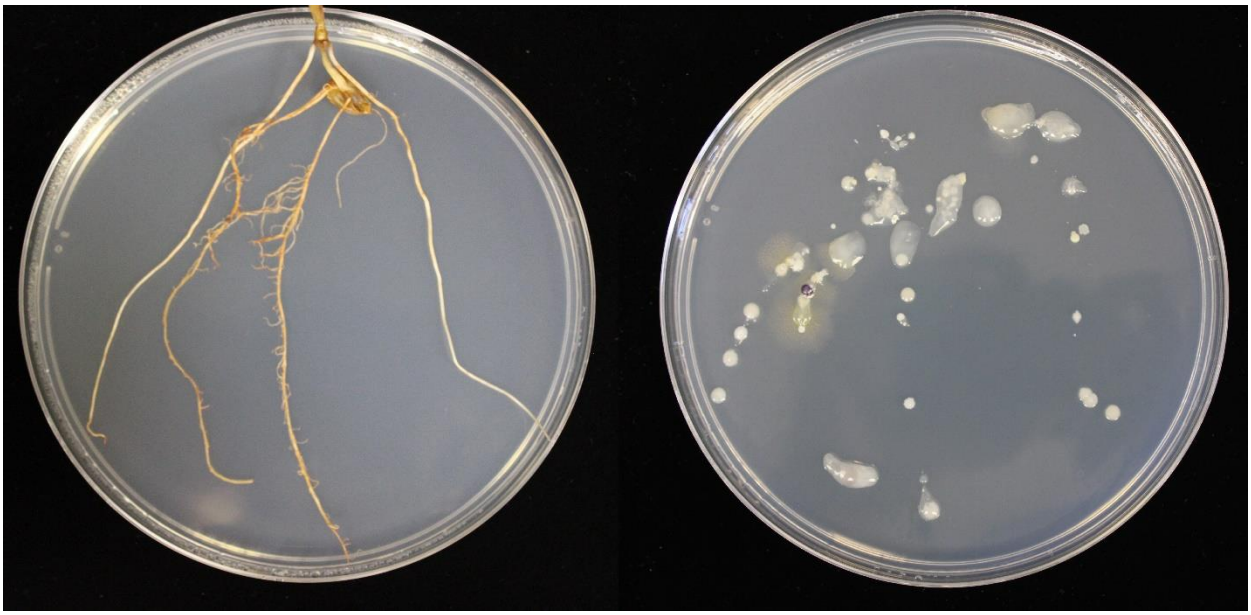

Figure 2.1 Root plating of maize roots.

Figure 2.2 Superimposed pictures of the root, the plate and colony growth.

Genomic characteristics

A total of 10 isolates had DNA of sufficient quality to be sequenced with Nanopore. Once these isolates were sequenced, they were then assembled and analyzed with TYGS. Phylogenetic analysis indicated suggested that seven are species that have not been previously undiscovered or unnamed (Table 2.1), using the criterion of an ANI score <95%. The bacteria corresponded to seven known genera, with Pseudomonas and Flavobacterium having more than one isolate each (Table 2.1).

Table 2.1 Taxonomic placement of the ten bacterial isolates.

Isolate	TYGS New Species	Most Closely Species	ANI
Janthinobacterium sp. RP23-3	Yes	*Janthinobacterium tructae* SNU WT3	91.40%
Pseudomonas palleroniana RP23-4	No	*Pseudomonas palleroniana* LMG 23076	98.74%
Duganella vulcana RP23-6	Yes	*Duganella vulcania* FT81W	95.10%
Flavobacterium sp. RP23-7	Yes	*Flavobacterium araucananum* DSM 24704	85.38%
Pantoea sp. RP23-9	Yes	*Pantoea endophytica* 596	88.99%
Pseudomonas sp. RP23-10	Yes	*Pseudomonas fluorescens* DR398	98.16%
Pseudomonas glycinae RP23-17	No	*Pseudomonas glycinae* MS586	98.83%
Flavobacterium chungangense RP23-19	No	*Flavobacterium chungangense* LMG 26729	98.47%
Sphingomonas sp. RP23-29	Yes	*Sphingomonas taxi* ATCC 55669	85.87%
Luteibacter sp. RP23-35	Yes	*Luteibacter rhizovicinus* DSM 16549	80.51%

We were able to obtain complete assemblies of these genomes, only getting one contig for most isolates, except *Pantoea sp.* RP23-9 and *Sphingomonas sp.* RP23-29, both of which had two plasmids (Table 2.2).

Table 2.2 Genomic features of the ten bacterial isolates.

Isolate	Genome Size	Contigs	GC	L50
Janthinobacterium sp. RP23-3	6,309,404	1	63.0	1
Pseudomonas palleroniana RP23-4	6,332,180	1	60.5	1
Duganella vulcana RP23-6	7,310,790	1	64.7	1
Flavobacterium sp. RP23-7	5,443,835	1	33.7	1
Pantoea sp. RP23-9	5,122,522	3	54.6	1
Pseudomonas sp. RP23-10	6,479,542	1	63.0	1
Pseudomonas glycinae RP23-17	6,305,104	1	60.4	1
Flavobacterium chungangense RP23-19	5,617,177	1	33.8	1
Sphingomonas sp. RP23-29	4,134,141	3	68.3	1
Luteibacter sp. RP23-35	4,553,789	1	64.7	1

Gene content ranged from 4,423 genes in *Sphingomonas sp.* RP23-29 to 7,333 in *Duganella vulcana* RP23-6 (Table 2.3). In functional characterization with RAST, the percentage of genes assigned to subsystems (groups of genes that work together to perform a particular function) ranged from 17% of the genes in subsystems in *Flavobacterium chungangense* RP23-19 to 29% in *Pseudomonas palleroniana* RP23-4 and *Pseudomonas glycinae* RP23-17 (Table 2.3).

Table 2.3 Genomic annotation of the isolates.

Isolate	**Predicted Protein Coding Sequences**	**RNA Genes**	**Number of Subsystems**	**Subsystem Coverage**
Janthinobacterium sp. RP23-3	6,212	116	341	1,514 (25%)
Pseudomonas palleroniana RP23-4	6,514	89	380	1,888 (29%)
Duganella vulcana RP23-6	7,333	110	341	1,559 (22%)
Flavobacterium sp. RP23-7	5,595	86	269	980 (18%)
Pantoea sp. RP23-9	5,277	100	347	1,624 (31%)
Pseudomonas sp. RP23-10	6,095	91	385	1,669 (28%)
Pseudomonas glycinae RP23-17	6,666	92	378	1,882 (29%)

Table 2.4 Most similar genome in the RefSeq database and gene content comparisons between the isolates from the rhizoplane and their most closely related isolates.

Isolate	Most Closely Related Isolate	ANI	Shared Genes	Unshared Genes
Janthinobacterium sp. RP23-3	*J. sp.* HH102	94.49%	3,748 (47.13%)	4,205 (52.87%)
Pseudomonas palleroniana RP23-4	*P. palleroniana* LMG 23076	98.74%	4,946 (76.22%)	1,543 (23.78%)
Duganella vulcana RP23-6	*D. vulcania* FT81W	95.10%	3,931 (41.71%)	5,493 (58.29%)
Flavobacterium sp. RP23-7	*F. bizetiae* HJ-32-4	86.13%	1,447 (16.71%)	7,211 (83.29%)
Pantoea sp. RP23-9	*P. sp.* S18	88.99%	2,756 (38.37%)	4,427 (71.83%)
Pseudomonas sp. RP23-10	*P. fluorescens* DR398	98.16%	5,246 (82.05%)	1,148 (17.95%)
Pseudomonas glycinae RP23-17	*P. glycinae* MS586	98.83%	5,103 (72.93%)	1,894 (27.07%)
Flavobacterium chungangense RP23-19	*F. chungangense* LMG 26729	98.47%	4,046 (57.07%)	3,044 (42.93%)
Sphingomonas sp. RP23-29	*S. sp.* CV7422	98.79%	3,262 (73.22%)	1,193 (26.78%)
Luteibacter sp. RP23-35	*L. rhizovicinus* DSM 16549	80.51%	331 (3.79%)	8,392 (96.21%)

Individual isolates

Janthinobacterium sp. RP23-3 shares a most recent common ancestor with *Janthinobacterium sp.* HH102 *and Janthinobacterium agaricidamnosum* BHSEK (this strain is different from the type sequence of *Janthinobacterium agaricidamnosum*) (Figure 2.4). Average Nucleotide Identity (ANI) analysis suggests that this isolate is a new species, since it has an ANI score below 95% to both the most closely related type genome (*Janthinobacterium tructae ANI = 91.40*) and the strains it shares a most recent common ancestor with (Figure 2.4).

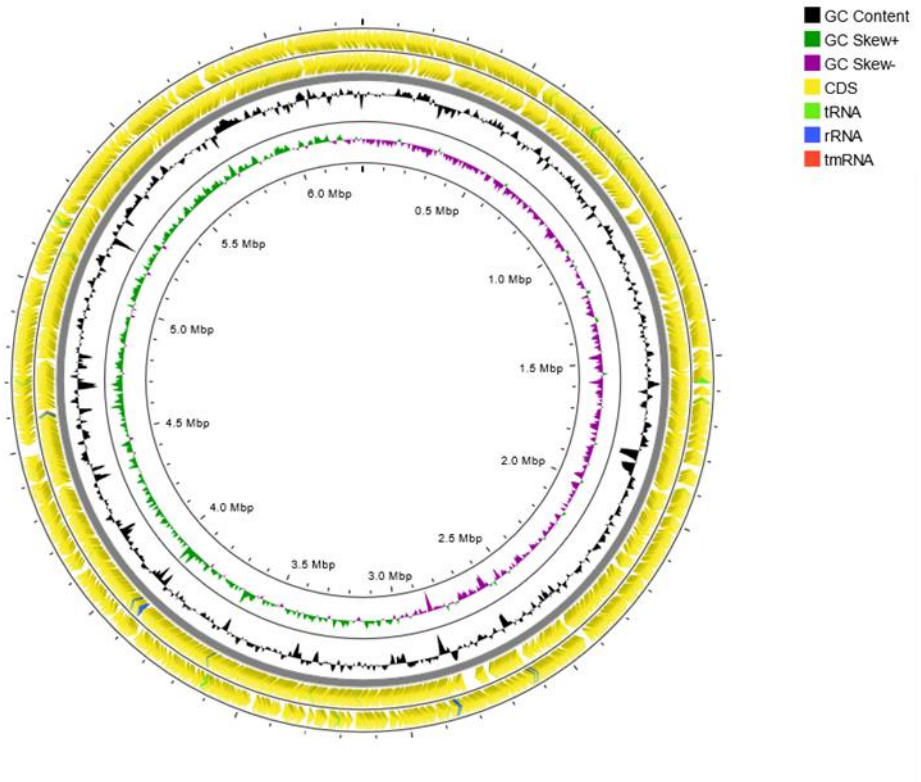

Figure 2.3 *Janthinobacterium sp.* RP23-3 chromosome.

Janthinobacterium sp. RP23-3 shares a most recent common ancestor with *Janthinobacterium sp.* HH102 and *Janthinobacterium agaricidamnosum* BHSEK (this strain is not related to the type sequence of *Janthinobacterium agaricidamnosum*) (Figure 4). Average Nucleotide Identity (ANI) suggest this isolate is a new species, since it is below 95% of both the most closely related type genome (*Janthinobacterium tructae* ANI = 91.40) and the strains it shares a most recent common ancestor (Figure 2.4).

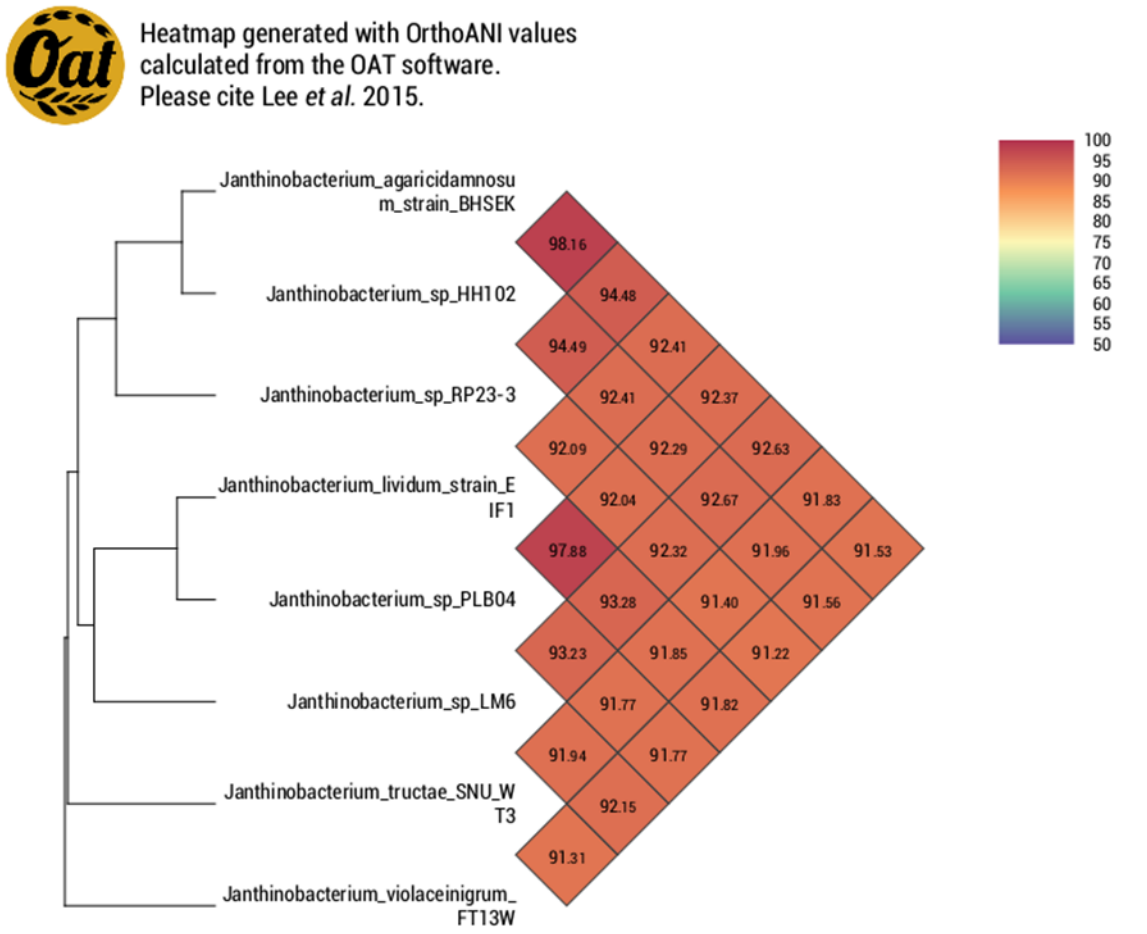

Figure 2.4 Comparisons of average nucleotide identities (ANIs) among *Janthinobacterium* strains. Red block: ANI scores are greater than or equal to 95%; orange block: ANIs are less than 95%.

Pseudomonas palleroniana RP23-4, has a genome size of 6,332,180 base pairs, in a single chromosome (Figure 3.5), L50 and N50 are 6,332,180 and 1 respectively. GC content is 60.5% (Table 2.2). There were 6,603 protein-encoding sequences predicted in total, consisting of 6,514 protein-coding sequences and 89 RNA genes. Only 29% of the protein-coding sequences (1,888) for this genome could be grouped into the functional subsystems with the RAST SEED server (Table 2.3). Genes shared between *Pseudomonas palleroniana* RP23-4 and *Pseudomonas palleroniana* Q1 were 4,946 out of 6,489 (76.22%) (Table 2.4).

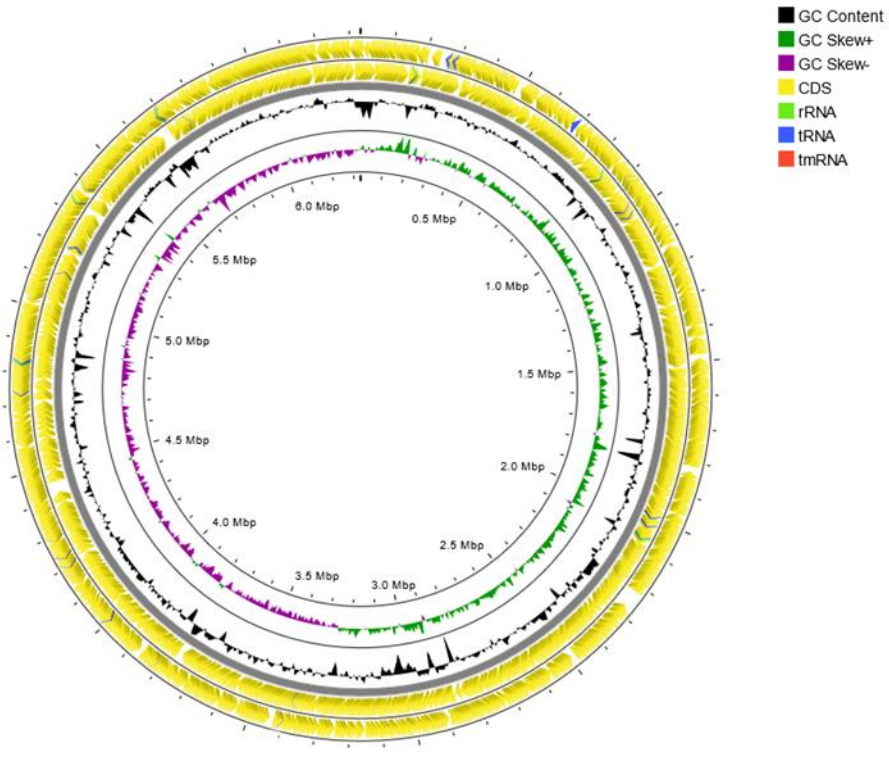

Figure 2.5 *Pseudomonas palleroniana* RP23-4 chromosome.

Pseudomonas palleroniana RP23-4 had an ANI score over 98% to all of the genomes of the *Pseudomonas palleroniana* strains currently present in the RefSeq database (Figure 2.6). The highest ANI score was to *Pseudomonas palleroniana* Q1, at 98.74% (Figure 2.6).

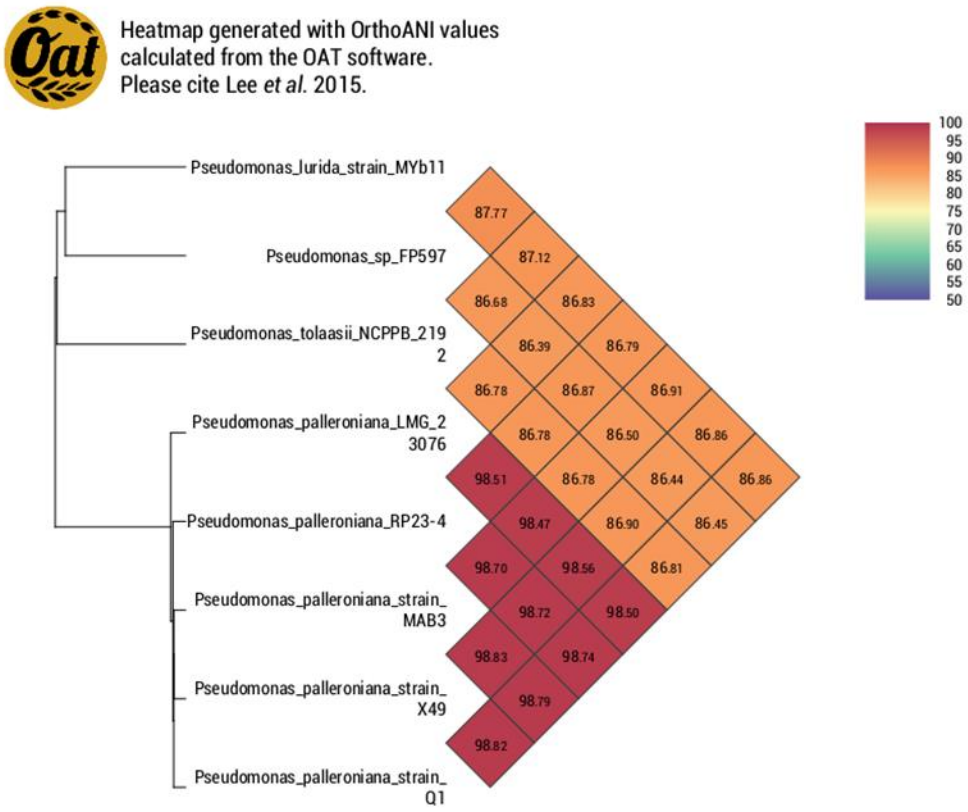

Figure 2.6 Comparisons of average nucleotide identities (ANIs) among *Pseudomonas palleroniana* RP23-4.

Duganella vulcana RP23-6 has a genome size of 7,310,790 (Figure 2.7) base pairs, in a single contig, L50 and N50 are 7,310,790 and 1 respectively. GC content is % (Table 2.1). A total of 7,443 coding sequences were predicted in *Duganella vulcana* RP23-6, consisting of 7,333 protein-coding sequences and 110 RNA genes (Table 2.3). Only 22% of the protein-coding sequences (1,559) for this genome could be grouped into the functional subsystems by RAST SEED server (Table 2.3). Genes shared between *Duganella vulcana* RP23-6 and *Duganella vulcana* FT81W were 3,931 out of 9,424 (41.71%), while 5,493 of these genes (58.29%) were unshared (Table 2.4).

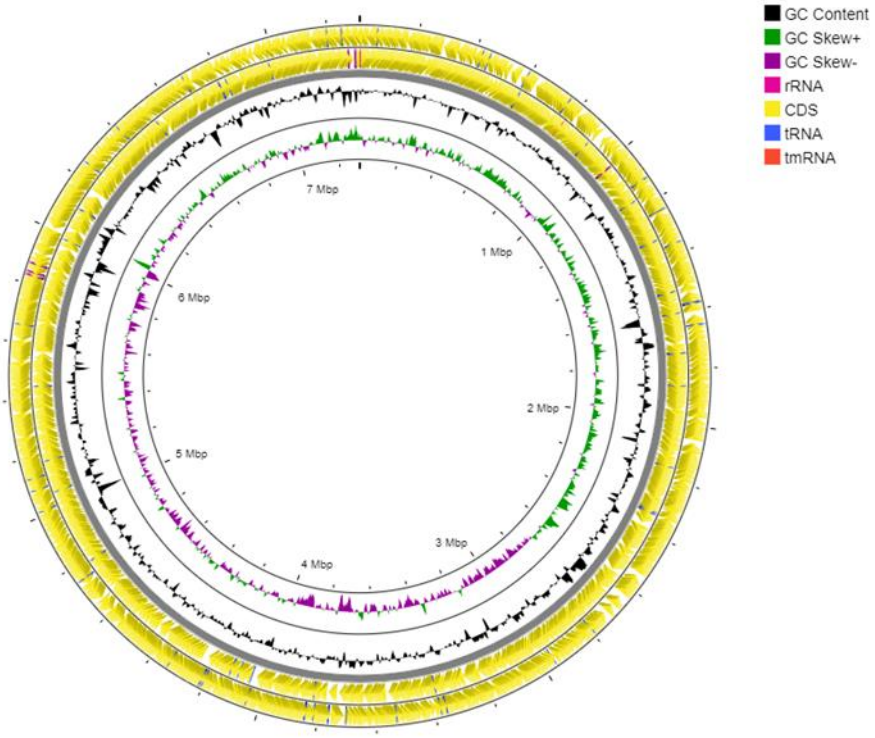

Figure 2.7 *Duganella vulcana* RP23-6 chromosome.

TYGS suggested *Duganella vulcana* RP23-6 as a potential new species (Table 2.1), and the Average Nucleotide Identity was 95.1% to the type genome of *Duganella vulcana* RP23-6 (Figure 2.8), so this isolate is borderline for a new species designation.

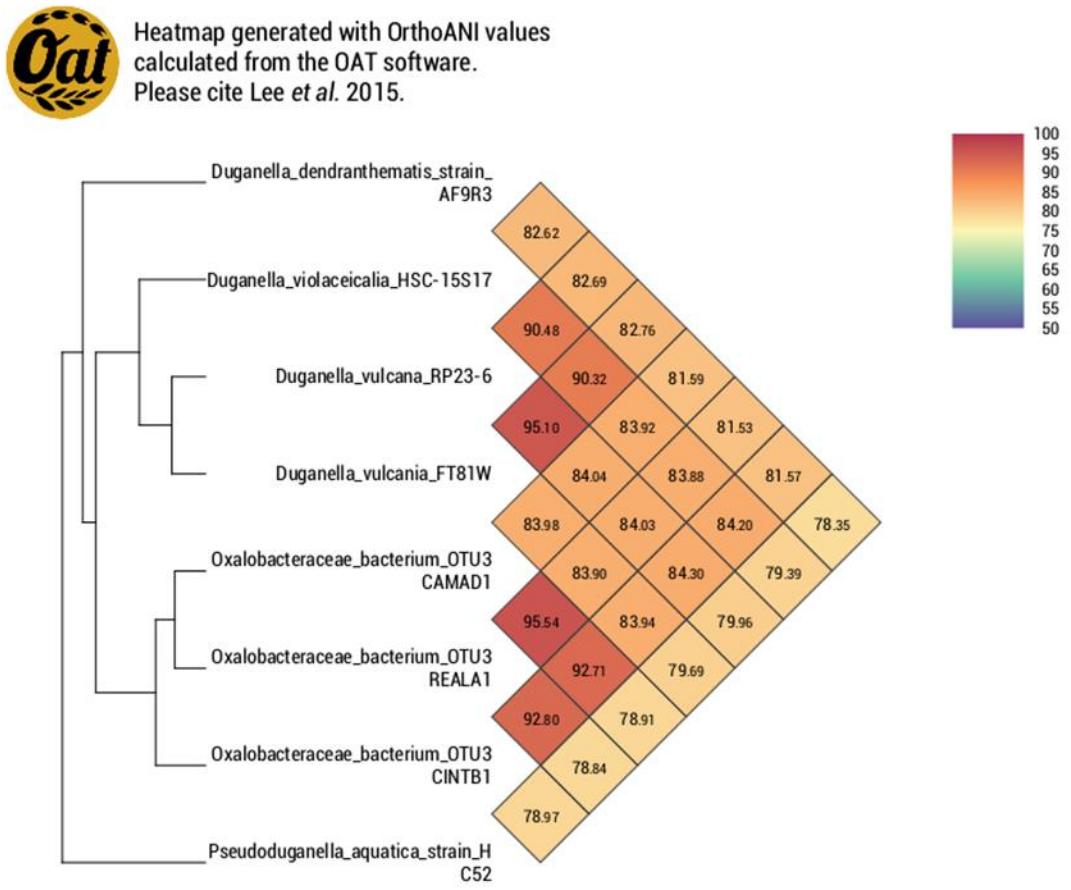

Figure 2.8. Comparisons of average nucleotide identities (ANIs) among isolates closely related to *Duganella vulcana* RP23-6.

Flavobacterium sp. RP23-7 has a genome size of 5,443,835 base pairs, in a single chromosome (Figure 2.9), L50 and N50 are 5,443,835 and 1 respectively. GC content is 33.7% (Table 2.1). There were 5,681coding sequences predicted in total, consisting of 5,595 protein-coding sequences and 86 RNA genes. Only 18% of the protein-coding sequences (980) for this genome could be grouped into the functional subsystems of the RAST SEED server. There were 1,447 (16.71%) genes shared between *Flavobacterium sp.* RP23-7 and its closest good relative with a complete genome sequence, *Flavobacterium sp.* KBS0721, while 7,211 (83.29%) were not shared.

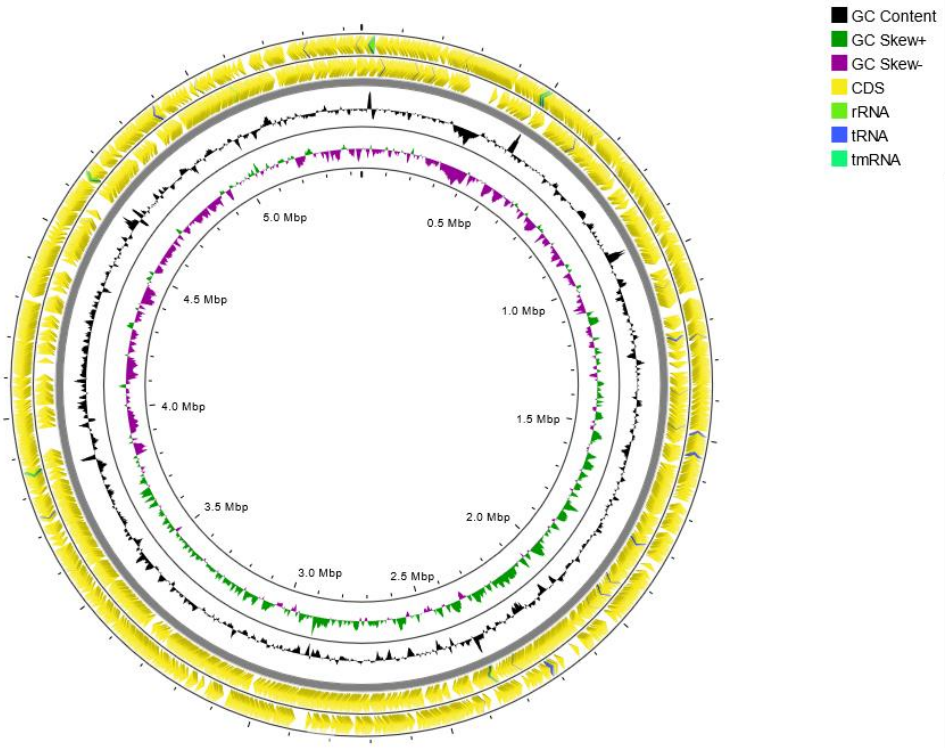

Figure 3.9. *Flavobacterium sp.* RP23-7 chromosome.

Flavobacterium sp. RP23-7 is in a clade with *Flavobacterium bizetiae*, *Flavobacterium sp.* KBS0721 and *Flavobacterium sp.* SBL02 (Figure 2.10). The ANI scores of these genomes are all below 90%. This result indicates that these are all different species, and possibly different genera (Figure 2.10). However, because there are species of *Flavobacterium* such as *Flavobacterium psychroterrae* (Figure 2.10), that are very distant to all other Flavobacteria, it seems appropriate to consider *Flavobacterium* as a very broad genus, and that our isolate is a new species within that genus.

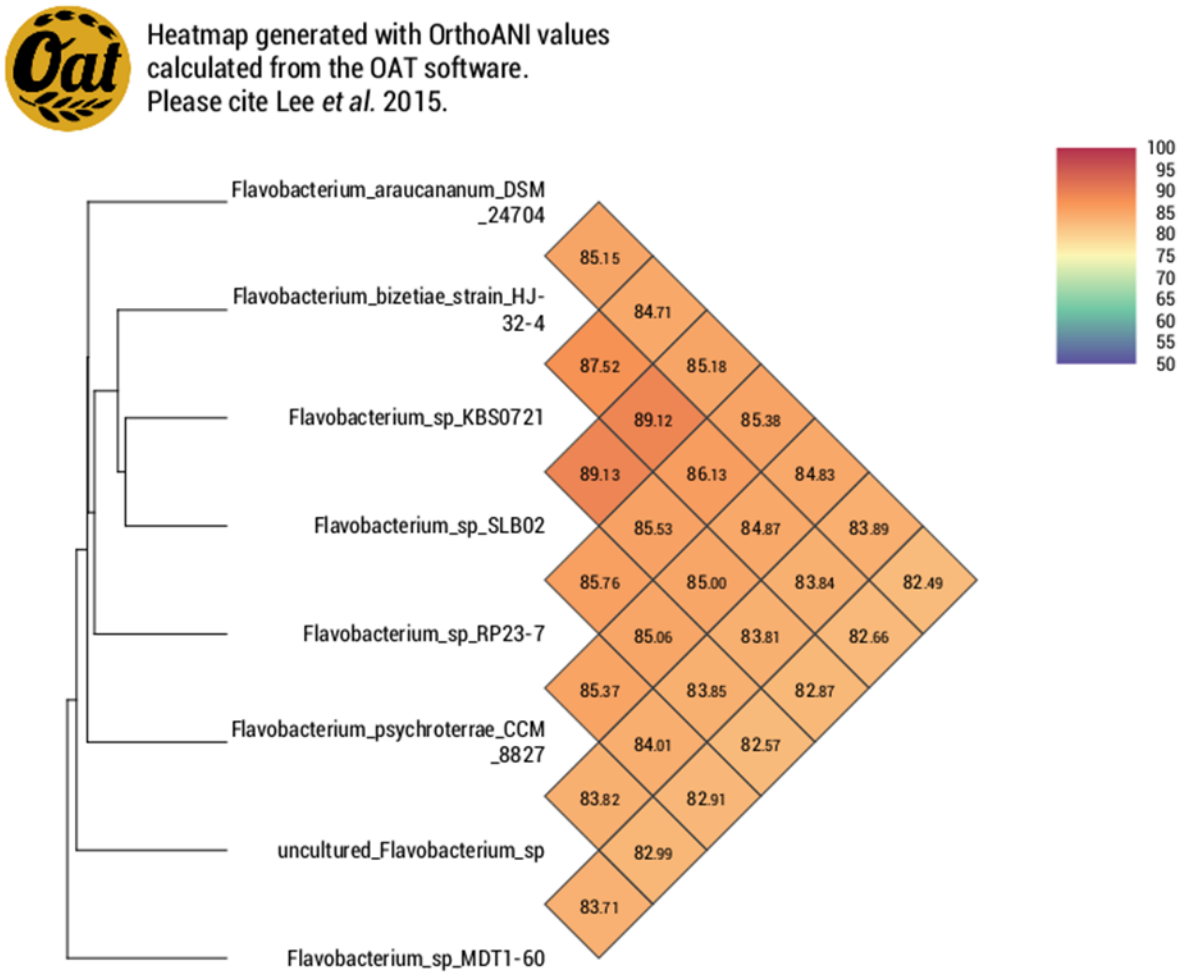

Figure 2.10 Comparisons of average nucleotide identities (ANIs) among isolates closely related to *Flavobacterium sp.* RP23-7.

Pantoea sp. RP23-9 has a genome size of 5,122,351 base pairs, in 3 contigs, including one chromosome of 4,201,854 (Figure 2.11) base pairs, and two plasmids of 836,081 and 84,416 (Figure 2.12) base pairs. L50 and N50 are 4,201,854 and 1 respectively. GC content is 54.6%, (Table 2.1), in the case of the chromosome, GC content is 54.7% while for Plasmid 1 and 2 is 54.2% and 57% respectively. There were 5,377 coding sequences predicted in total, consisting of 5,277 protein-coding sequences (4,249 in the chromosome) and 100 RNA genes. Only 35% of the protein-coding sequences (1,522) for the chromosome could be grouped into the functional

subsystems of the RAST SEED server (Table 2.3). *Pantoea sp.* RP23-9 shared 2,756 (38.37%) protein coding sequences with *Pantoea sp.* S18, 4,427 (71.83%) were not shared.

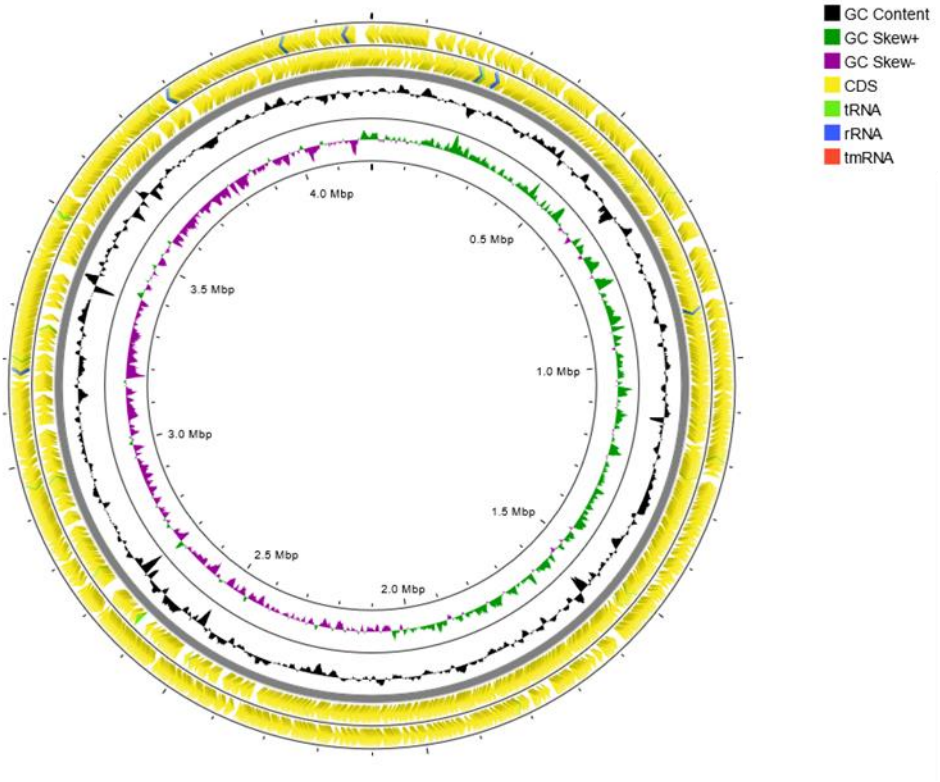

Figure 2.11 *Pantoea sp.* RP23-9 chromosome.

Plasmid 1 has a total of 899 predicted protein-coding sequences, only 102 of these (12%) were assigned to subsystems. Most of the genes assigned to subsystems are involved in amino acids and derivatives, carbohydrates, and stress responses. Plasmid 2 has a total of 129 predicted protein-coding sequences, none of which could be assigned to subsystems in RAST. Hence, the possible functions of the smaller plasmid are completely unknown and unpredictable. When compared to the ~31% predicted gene roles for the chromosomal gene complement, the emphasizes how little studied most plasmids have been.

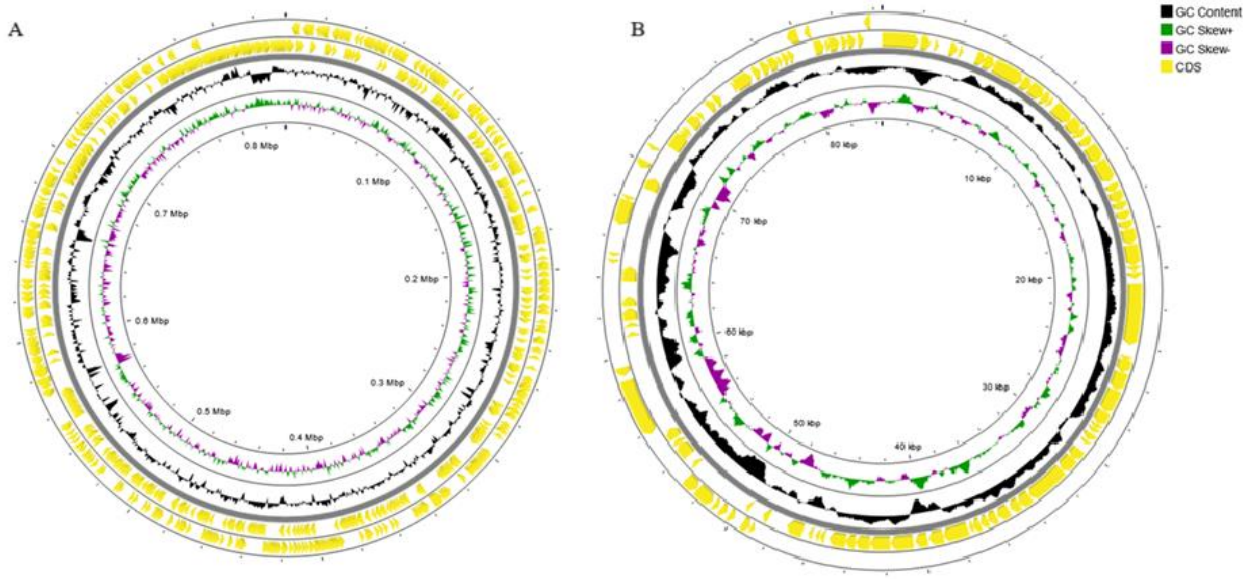

Figure 2.12 *Pantoea sp.* RP23-9 plasmids. (A) Plasmid 1 and (B) 2 Plasmid 2.

Pantoea sp. RP23-9 is in a clade with is in a clade with two species with well-sequenced genomes: *Pantoea endophytica* (the type genome and three strains with ANI > 98%), and two other isolates *Pantoea sp.* SOD02 and *Pantoea sp.* S18. The ANI between these six genomes range between 89.00% and 89.99% indicate that this is a new species (Figure 2.13).

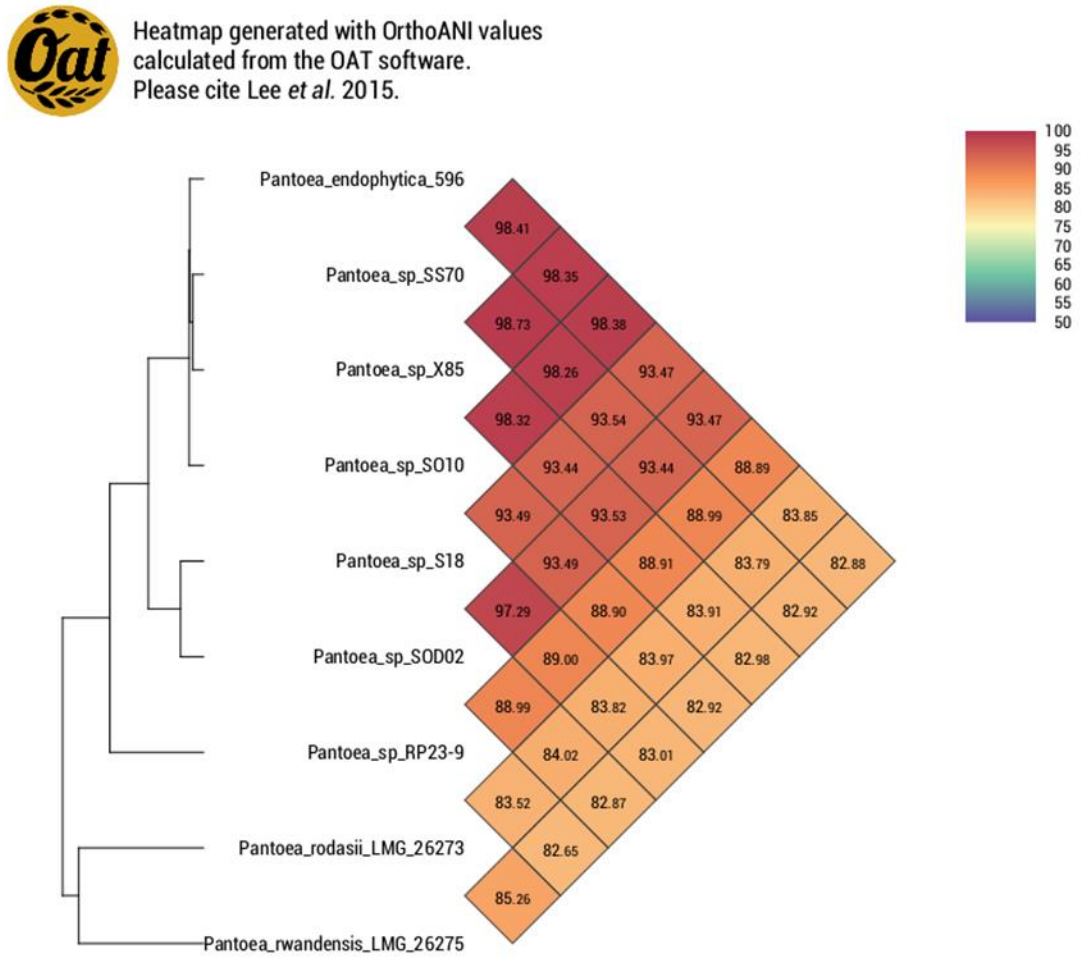

Figure 2.13 Comparisons of average nucleotide identities (ANIs) among isolates closely related to *Pantoea sp.* RP23-9.

Pseudomonas sp. RP23-10 has a genome size of 6,479,542 base pairs, in a single chromosome (Figure 2.14), L50 and N50 are 6,479,542 and 1 respectively. GC content is 63% (Table 2.1). There were 5,377 coding sequences predicted in total, consisting of 6,095 protein-coding sequences and 91 RNA genes. Only 28% of the protein-coding sequences (1,669) for this genome could be grouped into the functional subsystems of the RAST SEED server (Table 2.3). *Pseudomonas sp.* RP23-10 shared 5,246 (82.05%) genes with *Pseudomonas fluorescens* DR398, while 1,148 (17.95%) genes were not shared.

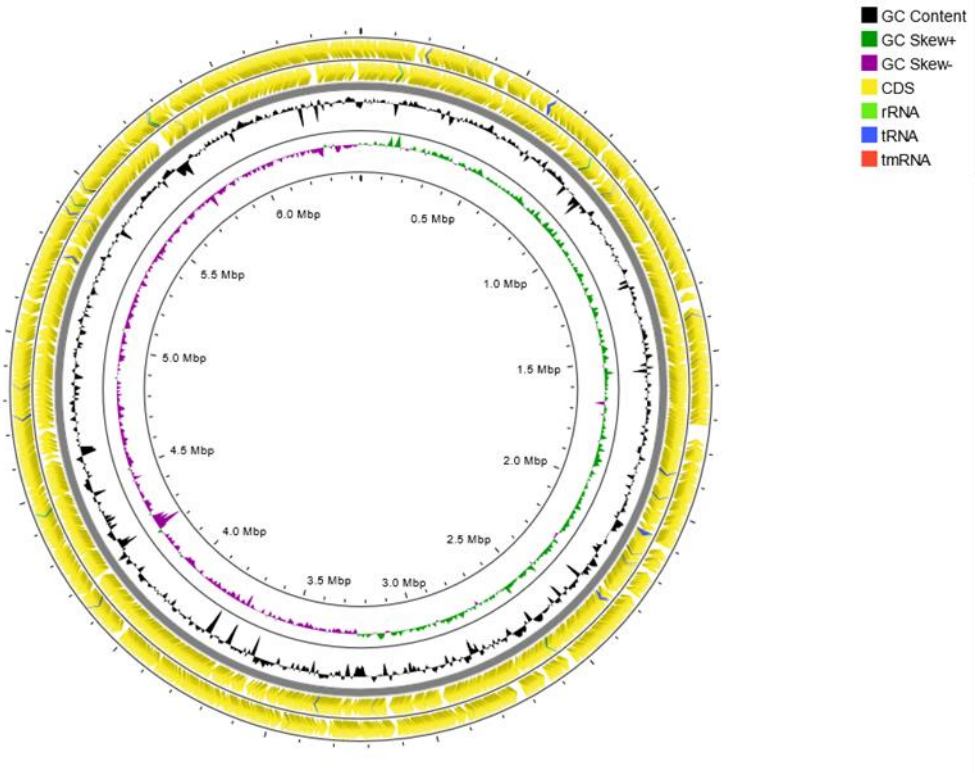

Figure 2.14 *Pseudomonas sp.* RP23-10 chromosome.

Pseudomonas sp. RP23-10 shares a most recent common ancestor with *Pseudomonas fluorescens* DR319. They are in a clade with *Pseudomonas sp.* DR 5-09. They all have ANI scores >95% suggesting they are the same species. However, the ANI to the most closely related type genome outside this clade (which includes *Pseudomonas glycinae* and a different *P. fluorescens*) is <95% suggesting that the R397, RP23-10, DR 5-09 clade represents a new species.

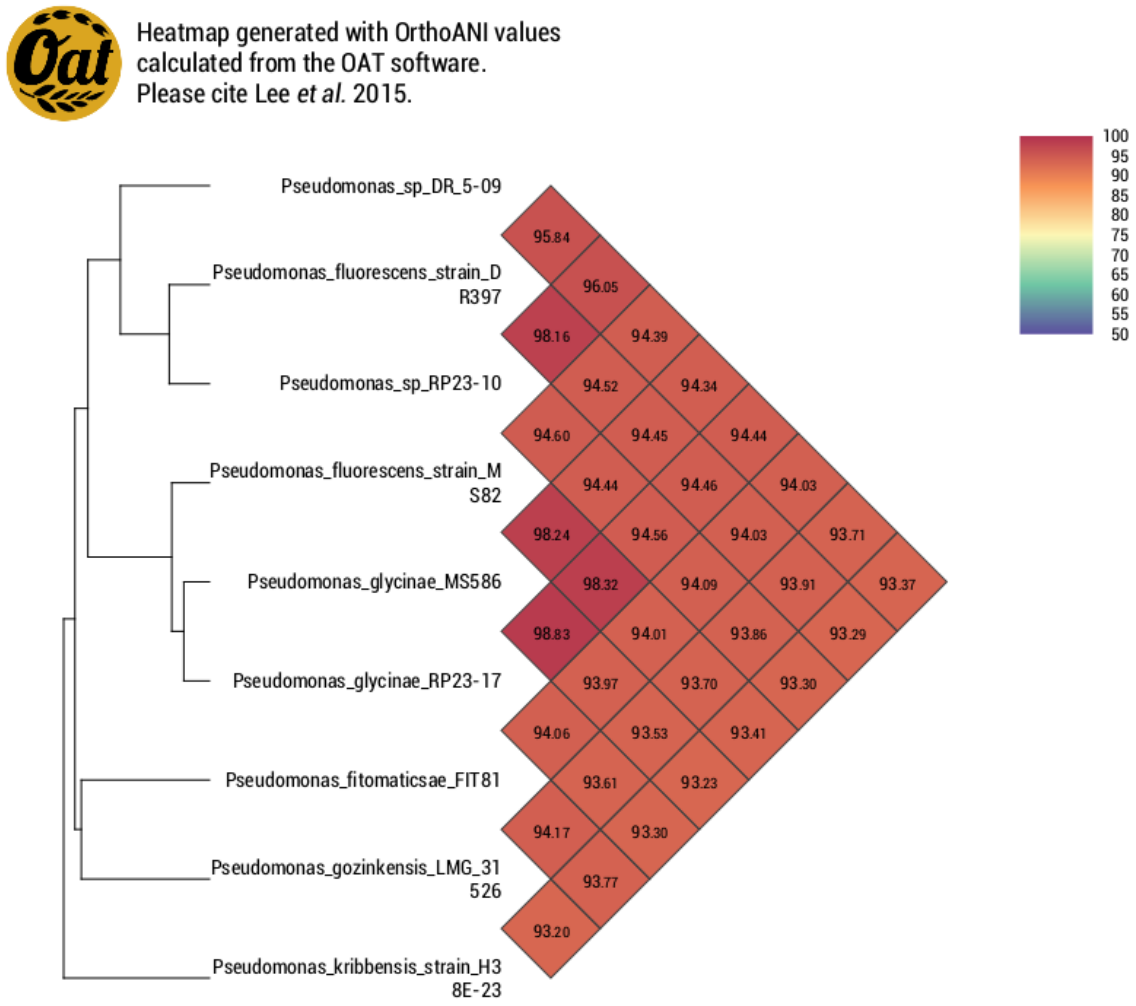

Figure 2.15 Comparisons of average nucleotide identities (ANIs) among *Pseudomonas* strains.

Pseudomonas glycinae RP23-17 has a genome size of 6,305,104 base pairs, in a single chromosome (3.16), L50 and N50 are 6,305,104 and 1 respectively. GC content is 60.4% (Table 2.1). There were 6,758 coding sequences predicted in total, consisting of 6,666 protein-coding sequences and 92 RNA genes. Only 29% of the protein-coding sequences (1,882) for this genome could be grouped into the functional subsystems of the RAST SEED server (Table 2.3). *Pseudomonas glycinae* RP-17 shared 5,103 (72.93%) genes with *Pseudomonas glycinae* MS586, while 1,894 (27.07%) genes were not shared.

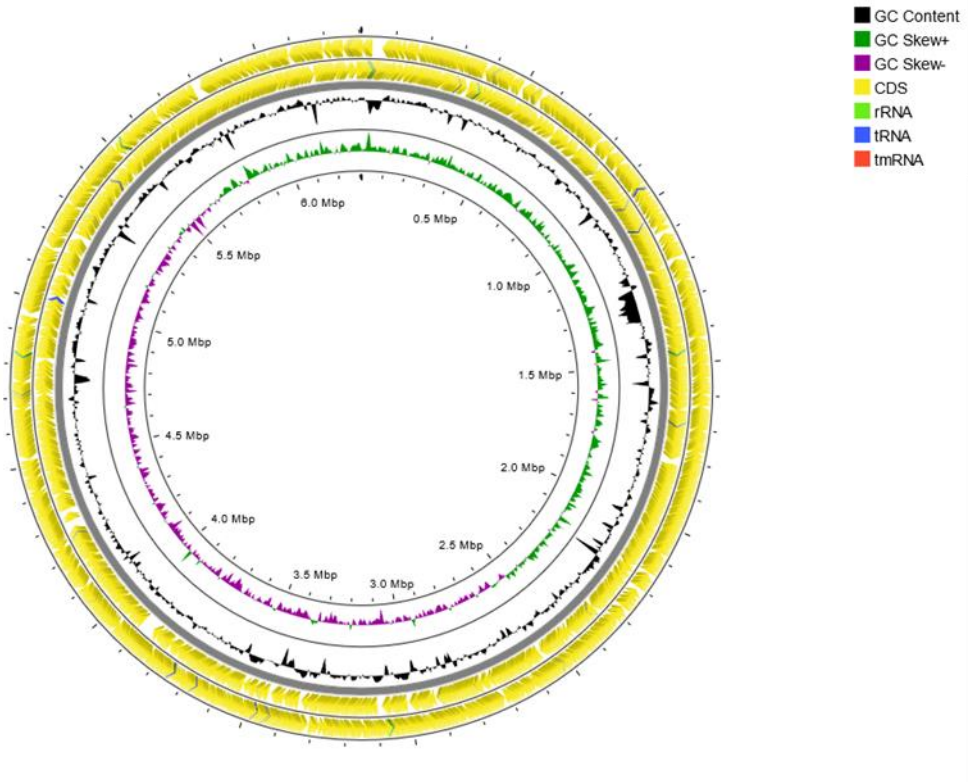

Figure 2.16 *Pseudomonas glycinae* RP-17 chromosome.

Pseudomonas glycinae RP23-17 is in a clade with the type genome for *Pseudomonas glycinae* MS586 and *Pseudomonas fluorescens* MS82, all of them have ANI >98% (Figure 2.16). This clade shares a most recent common ancestor with the clade where *Pseudomonas sp.* RP23-10, *Pseudomonas fluorescens* DR319, and *Pseudomonas sp.* DR 5-09 are (Figure 2.16).

Flavobacterium chungangense RP23-19 has a genome size of 5,617,177 base pairs, in a single contig, L50 and N50 are 5,617,177 and 1 respectively. GC content is 33.8% (Table 2.1). There were 6,785 coding sequences predicted in total, consisting of 6,704 protein-coding sequences and 81 RNA genes. Only 17% of the protein-coding sequences (1,137) for this genome could be grouped into the functional subsystems of the RAST SEED server (Table 2.3). *Flavobacterium chungangense* RP23-19 and *Flavobacterium chungangense* LMG 26729 shared 4,046 (57.07%) genes, while they had 3,044 (42.93%) unshared genes.

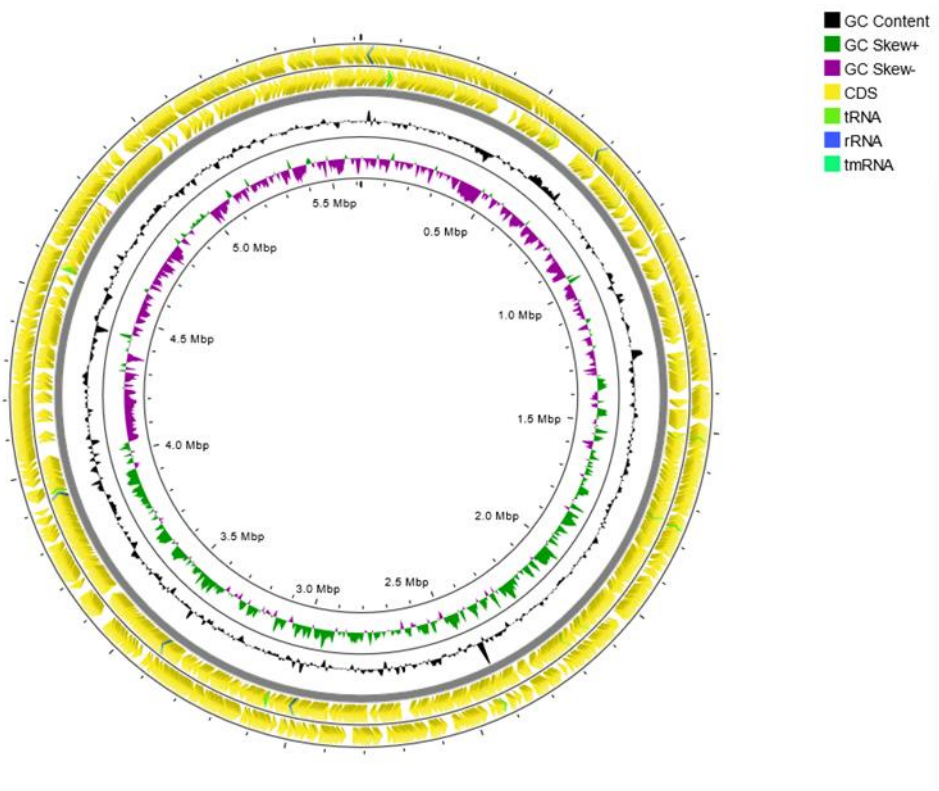

Figure 3.17 *Flavobacterium chungangense* RP23-19 chromosome.

Flavobacterium chungangense RP23-19 and *Flavobacterium chungangense* LMG 26729 have ANI scores of >98% (Figure 2.19). The closest genome to these two strains has ANI scores of 86% to these to these strains (Figure 2.19).

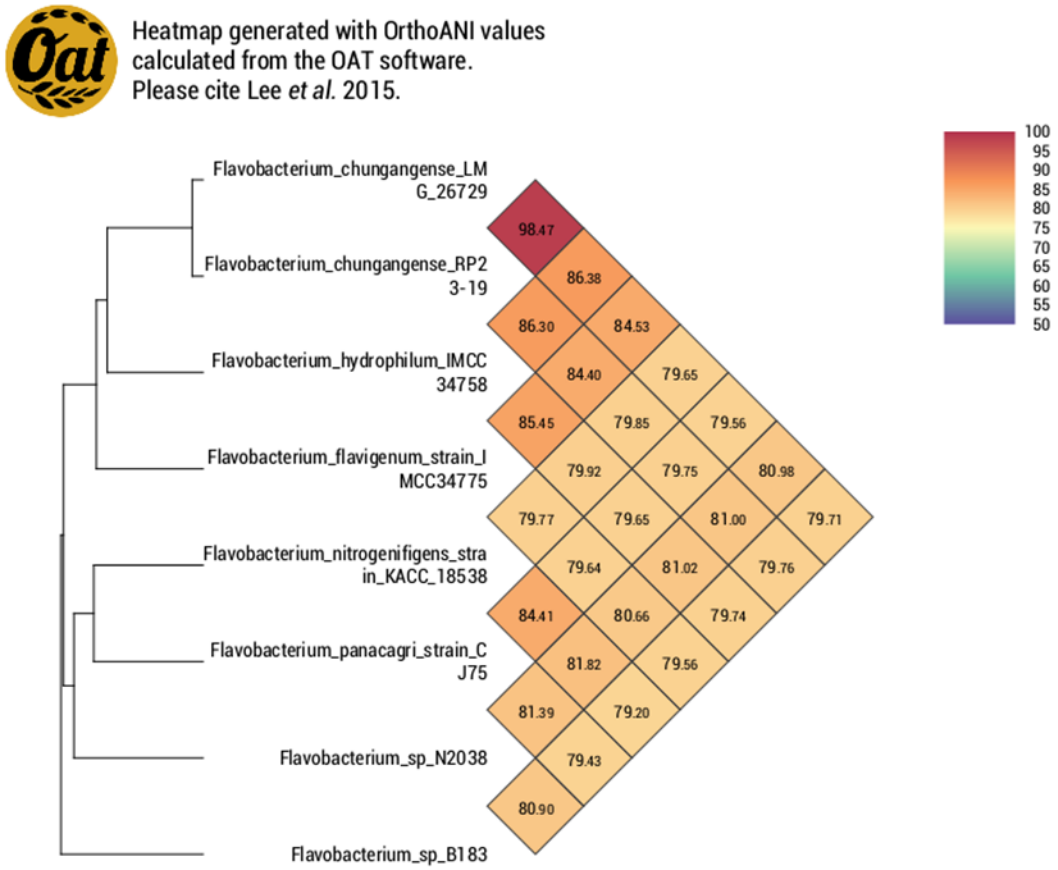

Figure 2.18 Comparisons of average nucleotide identities (ANIs) among isolates closely related to *Flavobacterium chungangense* RP23-19

Sphingomonas sp. RP23-29 has a genome size of 4,134,141 base pairs, in one 3,759,939 base pairs chromosome (Figure 2.19) and two plasmids: Plasmid 1 is 214,044 base pairs long and Plasmid 2 is 160,158 base pairs long (Figure 2.20). L50 and N50 are 3,759,939 and 1 respectively. GC content is 68.3% (Table 2.1). There were 4,485 coding sequences predicted in total, consisting of 4,423 protein-coding sequences and 62 RNA genes. Only 25% of the protein-coding sequences (1,104) for this genome could be grouped into the functional subsystems of the RAST SEED server (Table 2.3). *Sphingomonas sp.* RP23-29 and *Sphingomonas sp.* CV7422 have 3,262 (73.22%) in common, and 1,193 (26.78%) genes were not shared between the isolates.

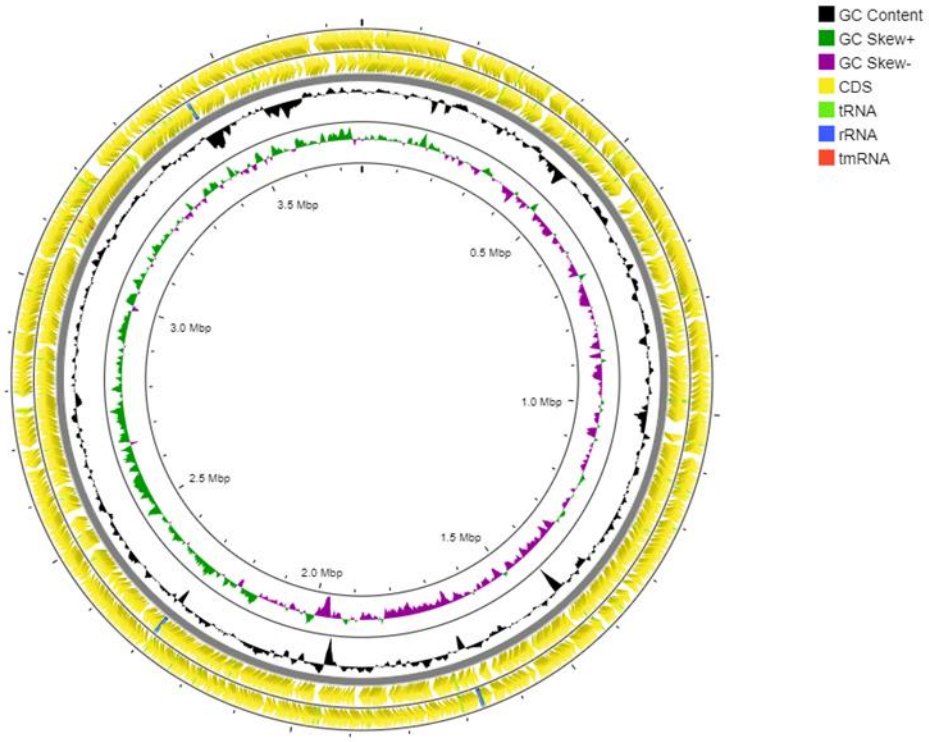

Figure 2.19 Chromosome of *Sphingomonas sp.* RP23-29.

The Plasmid 1 has a total of 228 coding sequences, only 10 of these coding sequences (4.38%) were assigned to subsystems. Most of the genes assigned to subsystems are involved in RNA metabolism, metabolism of aromatic compounds, carbohydrates, and resistance. Plasmid 2 has a total of 175 coding sequences, only 9 of these coding sequences (5.14%) were assigned to subsystems. The genes assigned to subsystems are involved in cell wall and capsule processes, protein metabolism and amino acids and derivatives.

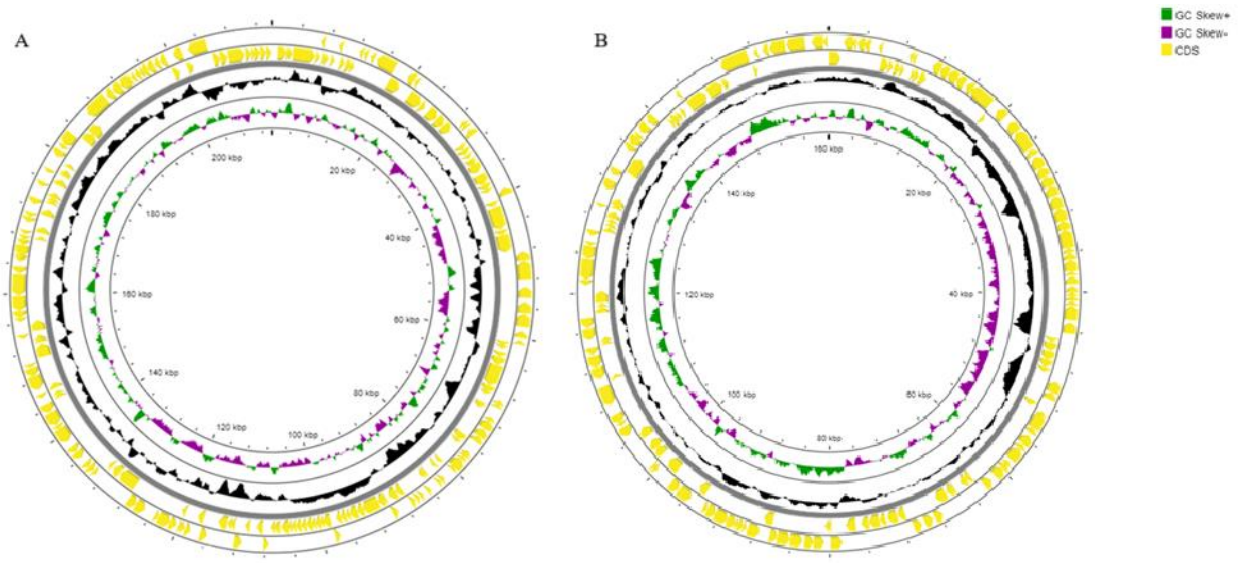

Figure 2.20 Plasmids of *Sphingomonas sp.* RP23-29. (A) Plasmid 1 and (B) Plasmid 2.

Sphingomonas sp. RP23-29 shares a most recent common ancestor with *Sphingomonas sp.* CV7422, and their ANI score is 98.97%, suggesting that they are strains from the same species (Figure 2.21). These two strains are in a clade with *Sphingomonas taxi*, the ANI score with this genome is 85%, suggesting that *Sphingomonas sp.* RP23-29 is an undescribed species.

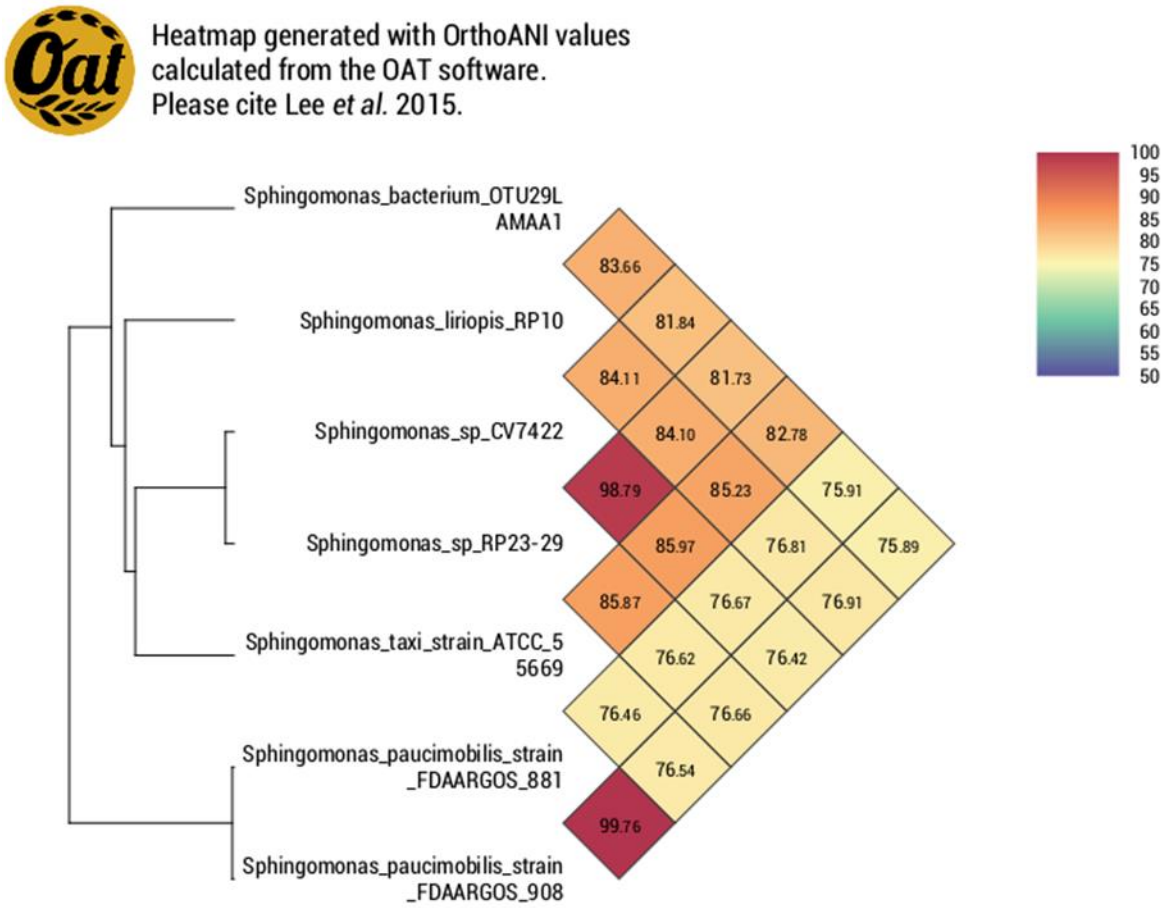

Figure 2.21 Comparisons of average nucleotide identities (ANIs) among isolates closely related to *Sphingomonas sp.* RP23-29.

Luteibacter sp. RP23-35 has a genome size of 4,553,789 base pairs, in one chromosome (Figure 2.22). L50 and N50 are 4,553,789 and 1 respectively. GC content is 64.7% (Table 2.1). There were 6,785 coding sequences predicted in total, consisting of 6,704 protein-coding sequences and 81 RNA genes. Only 17% of the protein-coding sequences (1,137) for this genome could be grouped into the functional subsystems of the RAST SEED server (Table 2.3). Only 331 (3.79%) were shared between the two isolates, while 8,392 (96.21%) were unique to each isolate.

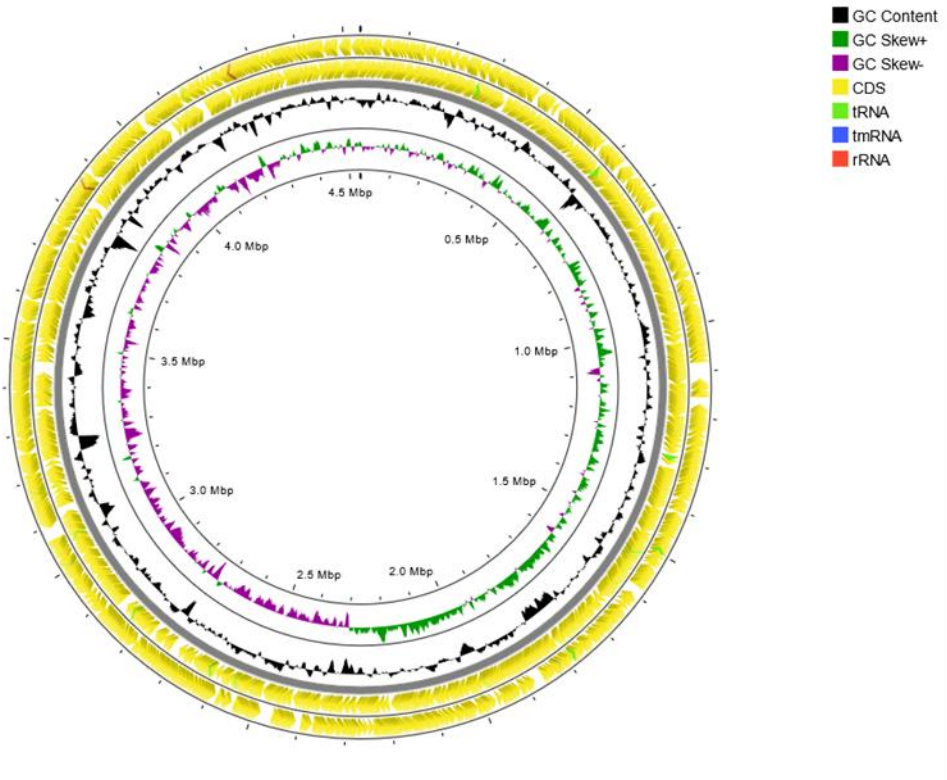

Figure 2.22 Chromosome of *Luteibacter sp.* RP23-35.

Luteibacter sp. RP23-35 shares a most recent common ancestor with *Luteibacter rizovicinus* (Figure 2.23). The ANI score between these two genomes is 80.51% indicating that this is a new species (Figure 2.23).

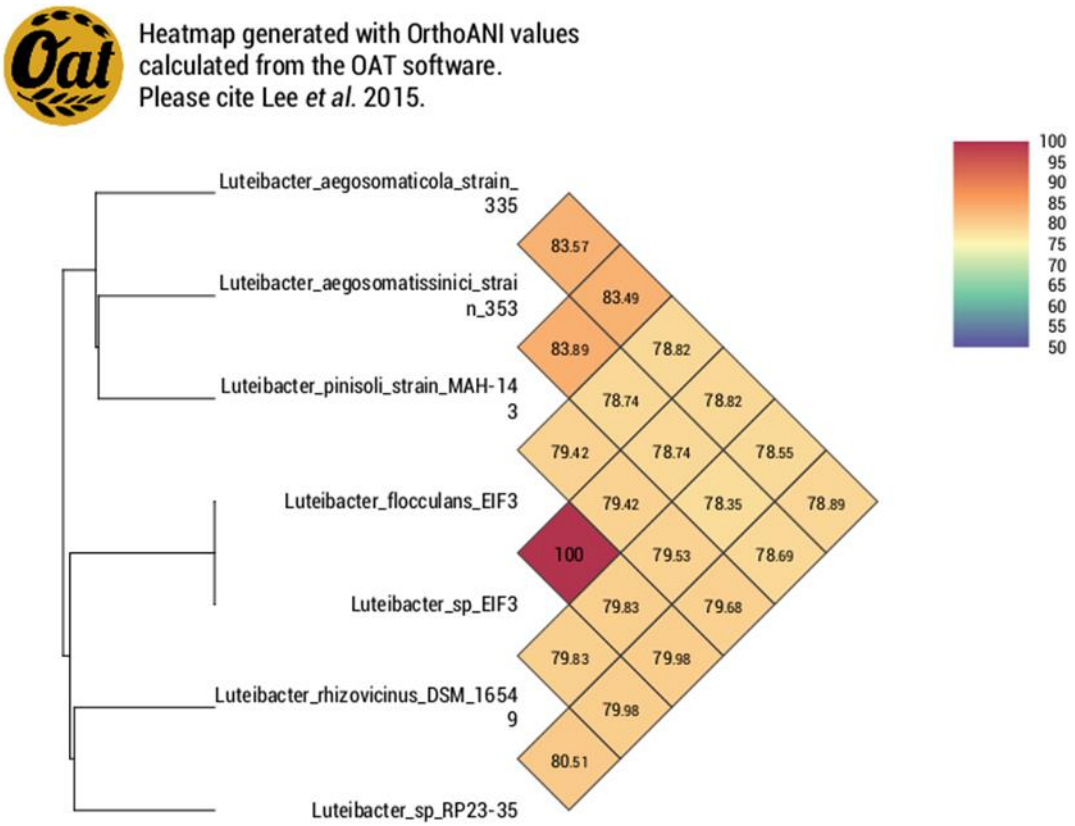

Figure 2.23 Comparisons of average nucleotide identities (ANIs) among isolates closely related to *Luteibacter sp.* RP23-35.

Gene content comparison

Gene contents (that is, gene number, but not specific gene function) in six subsystems were compared between the isolates and their closest relatives. These ten closest relatives were identified by BLASTn and ANI analyses and were selected as one closest relative per rhizoplane microbe. These closest relatives were isolated from a number of environments, but none from the root surface. Hence, this analysis is a first step toward characterization of the specific genetic properties that may be unusual to the rhizoplane environment. The subsystems compared were Carbohydrates, Motility and Chemotaxis, Iron acquisition and metabolism, Phosphorus metabolism, Sulfur metabolism, and Nitrogen metabolism.

Table 2.4 Average gene content in 6 RAST subsytems.

	Carbohydrates	Motility	Iron	Phosphorus	Sulfur	Nitrogen
Ten rhizoplane bacteria	258	81	20	31	28	18
Ten closest relatives	217	47	18	25	22	18
	p=0.0008	p=0.030	p=0.232	p=0.002	p=0.0091	p=0.678

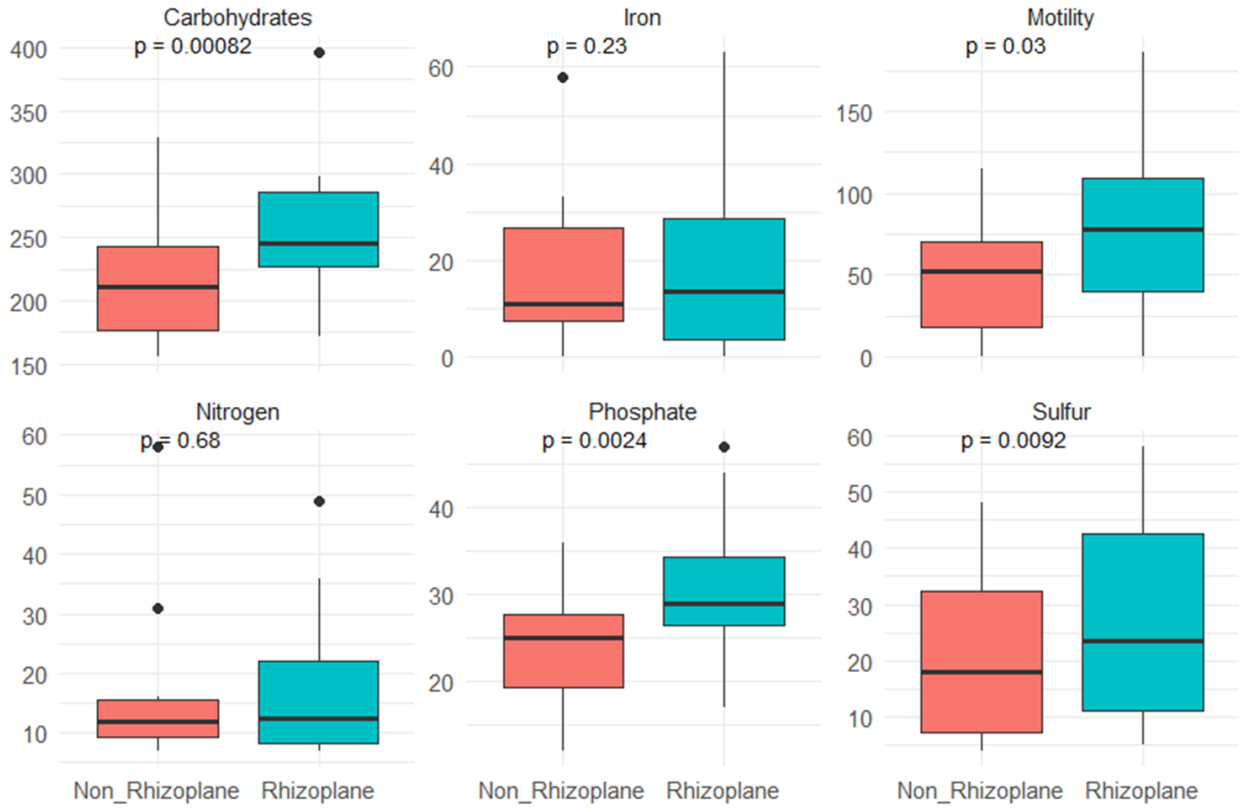

Figure 2.14 Boxplots looking at gene content in 6 RAST subsystems.

Subsystem analysis indicated that a few mineral metabolism-associated gene families were significantly enriched in the rhizoplane microbes, namely phosphorus and sulfur, but not including nitrogen or iron (Table 3.4, Figure 2.14). The most dramatic enrichment was for carbohydrate metabolism genes, perhaps reflecting carbohydrate exchange (and perhaps biofilm metabolism) between the microbes in this community and/or between the plant host and these microbes. The

enrichment in the rhizoplanes for motility-related genes was also significant, a result that is not surprising for organisms that were acquired because they were easily released from the root surface during the replica plating process.

Discussion

The rhizoplane plays a critical role in plant-microbe interactions, serving as a dynamic interface where plants and microorganisms exchange nutrients and signals. However, studying it presents challenges, particularly due to its separation from the endosphere and its lack of a physical, or metabolic, clear-cut separation from the rhizosphere. Typically, rhizoplane communities are removed from the root surface through chemical or mechanical treatments to study endophytes alone (Chaluvadi and Bennetzen 2018; Edwards et al. 2015; Richter-Heitmann et al. 2016). In this study, ten bacteria were isolated from the rhizoplane by replica plating the roots onto a petri dish, allowing for the isolation of bacteria present, but not completely attached, on the rhizoplane.

Janthinobacterium and *Duganella* belong to a group of 11 genera within the family Oxalobacteraceae of the Betaproteobacteria (Haack et al. 2016). Both genera, particularly *Janthinobacterium* and most *Duganella* species, are known for producing violacein, a bisindole compound. Violacein is a purple, indole-derived natural pigment with commercial potential due to its wide-ranging antibacterial, antiviral, anti-protozoan, and anti-cancer properties. *Janthinobacterium* strains are often linked to plant growth promotion and disease protection (Yin et al. 2021), while certain *Duganella* species exhibit plant-growth promoting traits, including the ability to solubilize phosphorus, potassium, and zinc in soils. Additionally, some *Duganella* isolates show antifungal activity, particularly against plant pathogens like *Fusarium graminearum* (Haack et al. 2016). While *Duganella vulcana* was first isolated from tropical streams in China (H.

Lu et al. 2022), further analyses and experiment with *Duganella vulcana* RP23-6 can help to increase the understanding of the effects of *Duganella* with plants. In the case of the most closely related isolates to *Janthinobacterium sp.* RP23-3, *Janthinobacterium sp.* HH102 was isolated from a rainwater-cistern in Germany (Haack et al. 2016) and there is no information regarding the isolation of *Janthinobacterium agaricidamnosum* BHSEK.

Pantoea is a bacterial genus containing numerous species that have been isolated from various environments (Walterson and Stavrinides 2015). Early studies in *Pantoea* primarily examined it's species that have parasitic associations with plants, including maize, cotton, melon, and onion (Walterson and Stavrinides 2015). Phytopathogenic strains of *Pantoea* cause a range of plant diseases, sometimes facilitated by factors like the type III secretion system (T3SS), quorum-sensing, and exopolysaccharides (EPS) (Walterson and Stavrinides 2015). Nevertheless, some *Pantoea* isolates have been found to enhance plant growth and provide protection against pathogens or environmental stressors (Duchateau et al. 2024; Lv et al. 2022; Walterson and Stavrinides 2015). The most closely-related described species to *Pantoea sp.* RP23-9 is *Pantoea endophytica*, which was isolated from maize tissues (Gao et al. 2019). However, the authors do not provide much information regarding which specific tissues or if it has any effect on the plants.

Plant-associated *Pseudomonas* species can act as saprophytes or parasites, colonizing plant surfaces and internal tissues (Preston 2004). While many strains enhance plant growth by suppressing pathogens, producing growth-stimulating hormones, and increasing resistance to diseases, some *Pseudomonas* strains inhibit plant growth, leading to issues like rot, necrosis, and galls (Preston 2004). The line between pathogenic and beneficial *Pseudomonas* is blurred because they often inhabit the same ecological zones and share similar methods for colonizing plants (Preston 2004). In fact, pathogenic, saprophytic, and beneficial strains often exist within the same

species, with disease severity influenced by environmental conditions and host-specific factors (Preston 2004). *Pseudomonas palleroniana* was originally isolated from rice, and was found to be mildly pathogenic (Gardan et al. 2002). There have been isolates which show both beneficial and pathogenic activities towards plants (S. Peng et al. 2023; Urón et al. 2018). In this study, the most closely related isolates to *Pseudomonas sp.* RP23-10 and *Pseudomonas glycinae* RP23-17, both *Pseudomonas fluorescens* DR398 (Nishu, No, and Lee 2022) and *Pseudomonas glycinae* MS586 (Jia et al. 2020), were isolated from the rhizosphere of soybean (*Glycine max*) in China. The case of *Pseudomonas fluorescens* DR398 (Nishu, No, and Lee 2022) shows some of the previously-reported broad diversity within the *Pseudomonas fluorescens* "species" group, since this genome is not closely related to the type genome of *Pseudomonas fluorescens* (Scales et al. 2014).

Flavobacterium is a genus within the phylum Bacteroidota that has not been extensively studied (Seo et al. 2024). Recent plant microbiome analyses have identified Bacteroidota as a prominent bacterial group in plant environments (Seo et al. 2024). Many species of *Flavobacterium* contribute positively to plant health by promoting growth, controlling diseases, and enhancing tolerance to abiotic stress (Seo et al. 2024). Despite these contributions, they have received less attention compared to more well-known plant growth-promoting rhizobacteria (PGPR) like *Pseudomonas* and *Bacillus* (Seo et al. 2024). Although some *Flavobacterium* species are recognized for their beneficial interactions with plants, the molecular mechanisms and bacterial factors driving these interactions are still not well understood, and the role of most *Flavobacterium* species in plant functioning is not well-described (Seo et al. 2024). Notably, many of the genomes from this genus have been sourced from aquatic environments, reflecting the historical focus on their pathogenicity in fish species (Seo et al. 2024). For example, of the three most closely related isolates to *Flavobacterium sp.* RP23-7 with comprehensive genome data, *Flavobacterium sp.*

KBS0721 was isolated from soil (NCBI), while *Flavobacterium bizetiae* and *Flavobacterium sp.* SBL02 are pathogens of aquatic organisms (Mühle et al. 2021; Petrushin, Belikov, and Chernogor 2020). Similarly, the type genome from *Flavobacterium chungangense* was isolated from a lake in South Korea (J.-H. Kim, Kim, and Cha 2009).

A few studies have looked at the growth promotion of *Sphingomonas*, especially at their plant growth promotion via the production of phytohormones and increased stress tolerance (Lombardino et al. 2022; Yang et al. 2014). Members of the genus *Luteibacter* have been previously isolated from the rhizosphere (Johansen et al. 2005), but their role in plant microbe interactions remains poorly understood.

Our method used for the isolation of the bacteria was very mild compared to earlier studies of root-surface microbiomes. One method used for isolating rhizoplane bacteria include suspending the roots in media (Turnbull, Liu, and Lazarovits 2012). Some studies have used sonication of washed roots to separate the rhizoplane microorganisms (Edwards et al. 2015; Richter-Heitmann et al. 2016), which would have variable levels of microbe release depending on the sonication intensity below a level that might rupture root cells and thus release endophytes (Richter-Heitmann et al. 2016). All these methods, including ours, requires that the isolated microbes have the potential to detach from the rhizoplane. This means that not all the bacteria in the rhizoplane are firmly attached to the root, and that different release technologies are likely to yield different subsets of the full rhizoplane community. Our technique of replica plating has the least violent disturbance of the root surface, so we expect that our released microbes are particularly weakly, and/or transiently, attached.

We have very little data regarding the attachment of our isolated microbes to the maize root, other than their presence and ease of release. However, the enrichment of genes

involved in mobility and chemotaxis in our isolate compared to their close relatives suggests that these species isolated are enriched for microbes that may travel along the rhizoplane surface, perhaps as founders of rhizoplane communities (Bonkowski et al. 2021; Knights et al. 2021).

Of the bacteria analyzed, only two were found to carry plasmids. Plasmids are genetic elements found outside the chromosome and are widespread in bacteria, are routinely transferred between bacteria of the same or different species (Shintani, Sanchez, and Kimbara 2015). Their genomes contain a mix of conditionally necessary genes, such as those for antibiotic or heavy metal resistance (Shintani, Sanchez, and Kimbara 2015). Plasmids also carry genes that are essential for plasmid functions like replication and mobility (Shintani, Sanchez, and Kimbara 2015). Long-read sequencing enables assemblies that can more easily identify plasmids, because assemblies from short-read sequences are often fragmented, making it challenging to reliably distinguish between plasmid and chromosomal sequences. The method used to identify plasmids in this project involved using circulator (Hunt et al. 2015) to circularize the assemblies and look into the annotations for plasmid genes. In all the cases, there was the parA gene present, which has been previously used to identify plasmids (Yagi et al. 2009). Notably, very few of the annotated genes on these two plasmids were assigned to known subsystems, highlighting the limited understanding of plasmid biology and the potential for discovering novel functions that are often observed with plasmids.

Bacterial gene content shows significant variability, which in comparative genomics is known to be associated with natural selection driven by different environmental pressures (Bolotin and Hershberg 2016). It has been noted that strains within the same species can differ by up to 30% in gene content, prompting questions about their classification within the same species (Konstantinidis and Tiedje 2005). These intraspecies variations are likely influenced by the strains'

differing ecological contexts, suggesting that a more ecologically focused and rigorous definition for prokaryotic species might be warranted (Konstantinidis and Tiedje 2005). Even genomes with extremely high similarity (ANI > 99.8%) can exhibit significant differences in gene content, up to approximately 10% of the total genes (Rodriguez-R et al. 2024). However, many of these differences are probably transient and involve genes that are not critical for metabolic or ecological functions in a particular environment (Rodriguez-R et al. 2024). Among our isolates, the differences in gene content between genomes with highest similarities (ANI > 98%) range between 18% and 43%. The difference in gene content between *Flavobacterium chungangense* RP23-19 and *Flavobacterium chungangense* LMG 26729 is certainly expected to be caused by the differences in their environments of origin. *Flavobacterium chungangense* LMG 26729 was originally isolated from a lake in South Korea, meaning that many of the unique genes in this strain may help the bacteria to adapt to the aquatic environment (J.-H. Kim, Kim, and Cha 2009).

Root-associated bacteria require specific genetic traits that enable them to adapt to the unique conditions of the root environment, and in some cases, they can provide benefits to the plant. The genomes of plant-associated bacteria often contain a greater number of genes related to carbohydrate metabolism (Levy et al. 2018), in the case of the genomes of the ten studied genomes, there was a significant difference in the number of genes associated with carbohydrate metabolism. This is expected, due to the bacteria in the rhizoplane having a close access to the carbon rich root exudates (Schmidt et al. 2018).

Genes involved in motility and chemotaxis are associated with rhizoplane colonization (Allard-Massicotte et al. 2016; De Weert et al. 2002; Knights et al. 2021), so it is to be expected that rhizoplane associated bacteria are significantly enriched for genes involved in motility and chemotaxis.

Due to the importance of siderophores (iron-chelating agents produced by numerous plant-associated microbes) for plant microbe interactions (Singh et al. 2022), the study also compared genes related to iron metabolism between rhizoplane and non-rhizoplane bacteria. However, no significant differences were found, suggesting that while iron acquisition is important for these microbes, it does not distinguish rhizoplane bacteria from their non-rhizoplane counterparts.

Phosphate-solubilizing bacteria transform soil phosphorus into forms that plants can absorb, making PSMs an important method for enhancing available phosphorus in (Pang et al. 2024). A variety of bacteria play a significant role in the phosphorus cycle, including processes like mineralization of organic phosphorus, dissolution of insoluble inorganic phosphorus, and phosphorus uptake (Pang et al. 2024). Since rhizoplane associated bacteria showed a significant enrichment in genes involved in phosphorus metabolism, it can be inferred that this is a trait for rhizoplane associated bacteria.

Sulfur is an essential nutrient for plants, absorbed by roots and processed in the leaves. It is critical for the synthesis of important amino acids like methionine and cysteine, which are essential for forming both structural and functional proteins (Bertoldo et al. 2021). Bacteria that associate with plants contribute to sulfur metabolism by primarily utilizing inorganic sulfate as a sulfur source, converting it into sulfide. This sulfide can then be absorbed by the plant to produce important sulfur-containing amino acids, such as cysteine and methionine (Andrés-Barrao et al. 2021).

Although the root microbiome is known to enhance nitrogen acquisition for plants, no significant differences in genes related to nitrogen metabolism were observed between the genomes of rhizoplane-associated and non-rhizoplane-associated bacteria (Trivedi et al. 2020).

This suggests that nitrogen acquisition capabilities are similar between these groups, regardless of their association with the rhizoplane.

In conclusion, this study provides valuable insights into the diversity and functional potential of rhizoplane-associated bacteria, highlighting both well-characterized and lesser-known genera involved in plant-microbe interactions. This study also found new species associated with this environment. Despite the known beneficial properties of some close relatives of our isolated bacteria, such as *Pseudomonas*, *Flavobacterium* and *Janthinobacterium*, many aspects of their interactions with plants remain underexplored. These results suggest that carbohydrate metabolism, motility and chemotaxis, phosphorus metabolism and sulfur metabolism are potentially important for interactions between the plant and the rhizoplane bacteria.

These novels, genome-characterized isolates represent a important advancement in the study of plant-microbe interactions. These isolates, analyzed at the genomic level, provide a unique opportunity for future research into the biological mechanisms involved in the interactions between plants and rhizoplane bacteria. By employing these isolates in experimental setups, we can explore various aspects of how rhizoplane bacteria can influence plant growth, nutrient uptake, disease resistance, and overall plant health.

CHAPTER 3

MICROBIAL PARTNER (MIPNER) ANALYSIS

Introduction

All organisms pursue their life histories in the presence of other biological forms, some as competitors, some in a prey-predator relationship, some as co-operators, including symbionts and other types of co-conspirators, mostly unnamed. Microbes, in particular, commonly exist in highly complex communities, surrounded by hundreds to millions of other species and races of microbes, in such diverse and unstable environments as the atmosphere, bodies of water, multicellular hosts, and the soil. Despite this known ubiquitous and dynamic complexity, traditional microbiological research has concentrated on the study of one purified microbial species at a time to conduct hypothesis-based research, where the effects of a single variable are assayed in the presence of overall constancy. The advent and fabulous ongoing enrichment of "OMICS" technologies over the last 30 plus years (Adams et al. 1991; Patterson and Aebersold 2003; Venter et al. 2004; Weckwerth 2003) has provided a fully reversed perspective from the "one microbe at a time" strategy to an "everything at once" approach. This has facilitated a rapid expansion in the study of microbial ecology, especially to examine whole community microbiome interactions and to explore multi-taxa ecosystem-level interactions (Chaudhry et al. 2021; Crandall et al. 2020; Morales and Holben 2011). The great wealth of data from OMICs approaches can provide deep and detailed correlations but come with their own weaknesses and limitations to interpretation. For instance, the enormous quantities of data and number of comparisons that are made always present statistical challenges, such as low resolving power and routine false positives, that must be

resolved (Carr et al. 2019; Storey and Tibshirani 2003). Hence, any correlations resulting from such analyses require further "hypothesis-driven" validation. With microbes, a more realistic environment for such confirmation experiments would require more than just the participation of a single microbial species. In recent years, many research groups have attempted to create reproducible (that is, somewhat stable) microbial communities that are simpler versions of real-world assemblages (Coker et al. 2022; Großkopf and Soyer 2014; Johns et al. 2016; Martins et al. 2023; McClure et al. 2020; Mee et al. 2014; Niu et al. 2017; van Leeuwen et al. 2023; Yin, Hagerty, and Paulitz 2022; Zengler et al. 2019). The problems that must be overcome include microbial competition, very different growth rates, antimicrobials, incompatible metabolic properties, and dissimilar environmental requirements (Johns et al. 2016). We propose that a simpler type of relatively stable microbial community can be created with self-identifying microbial partners. The simplest of these partnerships, with two members, would be tremendously less complex than the real world, but much more complex than a single species experiment. It is unlikely that we will be able to fully conceptualize a complex natural environment until we begin to understand binary microbial interactions. Such binary studies do exist, though many are consigned to the exploration of syntrophic interactions in co-culture (Shou, Ram, and Vilar 2007). However, some have provided field-relevant and fascinating information regarding microbial competition as a tool for resistance against root diseases (Thomashow 1996), for explaining how a soil bacterium can protect a soil fungus (Dahlstrom and Newman 2022), or in the formation, function and/or destruction of biofilms (Banks and Bryers 1991; Breugelmans et al. 2008; Nielsen et al. 2000). These previous examples all shared intense pursuits by dedicated research teams to investigate the biology of an "important" microbe. We believe that all microbes are worthy of investigation, and that the most important discoveries may come from microbes that we do not currently know

anything about. Hence, a more general and facile method for identifying microbial partnerships would provide a useful step forward. Here we present a novel method for isolating pairwise Microbial Partners (MiPners) from natural systems based on their propensity to physically associate with one another through microbe-microbe binding. Isolated partnerships can then be used to conduct hypothesis-based experiments. We have validated this method using a strain of *Serratia marcescens* isolated from Georgia soil, which was found to select and grow with only a tiny subset of the soil microbial collection, including with at least one bacterial strain that was unable to grow in the absence of its *S. marcescens* partner.

Methods

Isolation and characterization Serratia marcescens *strain, SMC43 (MiPner bait)*

As part of the course GENE4240L (Spring of 2021), students collected soil samples near the parking lot of Davison Life Sciences Complex on the UGA campus in Athens, GA (GPS coordinates: 33°56’ 35.8’’ N, 83°22’ 23.1’’ W). Soil suspensions were generated through mixing soil with phosphate buffered saline solution (PBS) from which 10^{-2}, 10^{-3}, and 10^{-4} dilutions were made with PBS. 100 microliters of each of the dilutions and the undiluted soil suspension was spread onto Soil Extract Agar (HiMedia Laboratories) plates. Plates were grown for two days at room temperature. Specific colonies were isolated to pure culture through several rounds of streaking and re-culturing on soil extract plates. Students Mary Norris and Molly Levin identified a red colony that they purified by several rounds of streaking (SMC43). DNA was extracted from an SMC43 overnight liquid culture of Nutrient Agar (Difco) using a protocol for high molecular weight DNA extraction (Mayjonade et al. 2016). Libraries were prepared using the Rapid Barcoding kit (SQK-RBK004, Oxford Nanopore) and sequenced for 72 h using R9.4.1 flow cells (FLO-MIN106, Oxford Nanopore) on a GridION instrument. The genome was assembled with

Canu using the default settings (Koren et al. 2017). The contigs were circularized using Circlator with the default settings (Hunt et al. 2015). The assembly was taxonomically analyzed with Type (Strain) Genome Server (TYGS) (Meier-Kolthoff and Göker 2019). The genome was annotated using RAST and Prokka (Aziz et al. 2008; Seemann 2014). A BRIG chart was constructed and using Proksee (Grant et al. 2023).

MiPner and Control Experiments.

To isolate MiPners of SMC43, we conceived a series of steps that required both cell-cell binding of environmental bacteria to SMC43 and subsequent growth with SMC43 on 0.1X Nutrient Agar plates. The first component is a cultured microbial bait, in this case SMC43. The second component is a soil suspension from which MiPners can be captured through their physical associations with the bait microbe. A set of control experiments were also designed to ensure that cultured bacteria were indeed due to associations between field-sourced bacterial communities with the SMC43 bait, and not due to other factors, including lab contamination or natural binding capacity to the bait sticks used. The full MiPner and control experiment strategy is detailed in Figure 3.1.

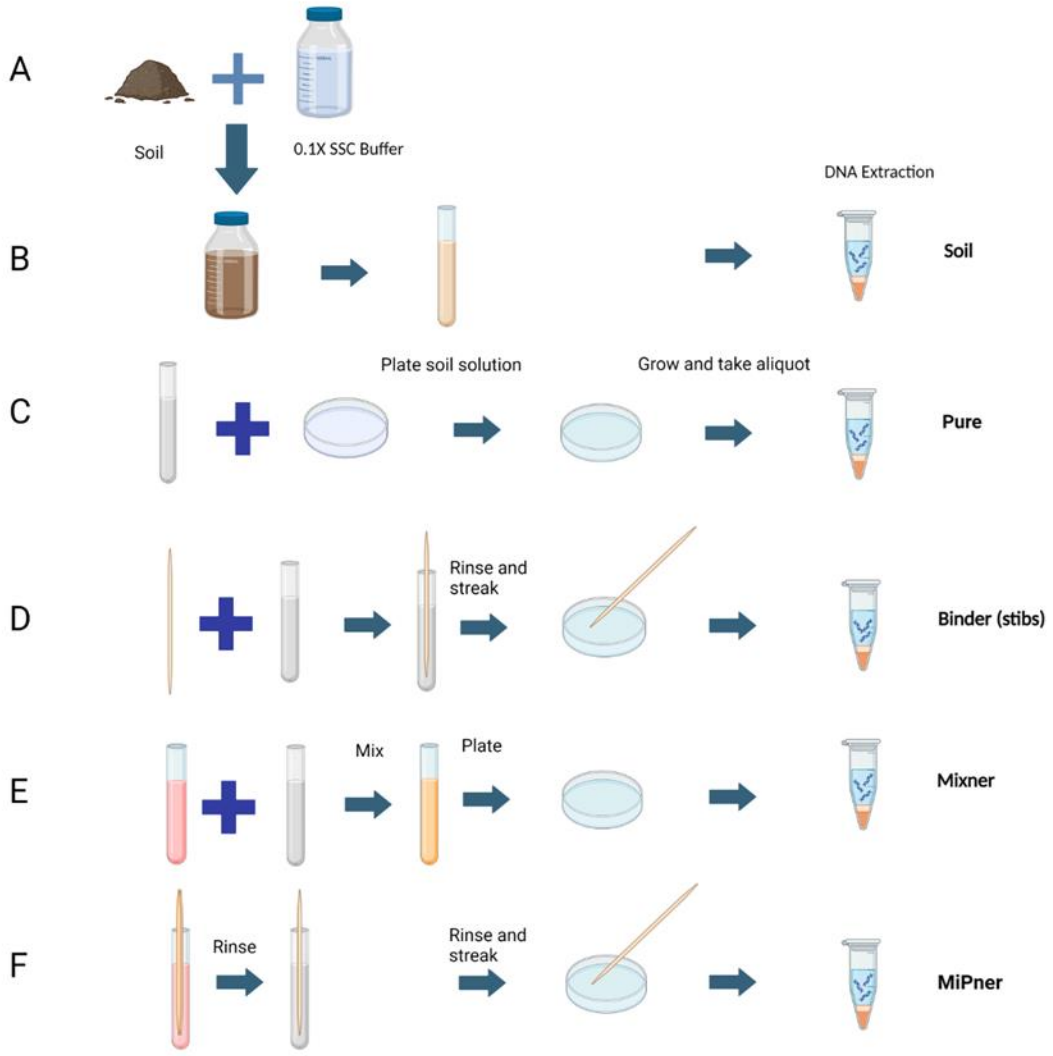

Figure 3.2 Flow chart describing MiPner steps and controls. MiPner steps and controls. A) Starting soil and buffer; B) The soil suspension is created from mixing the soil and buffer, allowing this to settle, and aliquoting only the supernatant (Soil); C) Microbes are plate cultured from the soil suspension (Pure); D) A wooden applicator is submerged into the soil suspension and used for plate culturing of microbes (Binder); E) Microbes are plate cultured from the soil suspension mixed with SMC43 (Mixner); F) A wooden applicator is submerged into the SMC43 solution, followed by submersion in the soil suspension, and used for plate culturing of SMC43-bound microbes (MiPner).

To characterize and quantify the 'source' communities from which MiPners would be picked, 2 ml aliquots of soil suspension were repeatedly transferred to tubes and centrifuged at 10,000 g for 10 minutes to precipitate bacteria and other microbes. Once 250 mg of precipitate had been collected, DNA was extracted from the pellet using a DNAeasy Power Soil Kit following the manufacturers protocols (Qiagen, Hilden, Germany) (Figure 3.1B). This collection process was carried out in triplicate to generate three technical replicate samples from the soil suspension. This is an important step in MiPner determination because bacteria found in the MiPner experiments but not in the source community may be from environmental contamination.

A second set of control experiments were carried out to identify how culturing methods and conditions could intrinsically bias the observed community when culturing both with and without SMC43. These experiments again act as controls to ensure that putative MiPners identified were indeed present within and culturable from the source community and not contaminants. For the first culture control (labeled 'Pure'), the soil suspension was used as an inoculant for direct culturing on 0.1 X Difco plates. 50 ul of the suspension was pipetted onto each plate across three technical replicates and spread using sterile glass beads. Plates were left for three days at room temperature (approximately 22 °C). To capture the communities from the plates for DNA extraction, 1 ml of 0.1 X SSC was added to the plate and gently shaken for five seconds, poured off into a tube, and then used as input for DNA extraction using the protocol of Mayjonade et al. (2016) (Figure 3.1C). All subsequent plates described were cultured and extracted in this manner unless otherwise specified.

The second culture control (Mixners) was used to identify which bacteria could survive or thrive in growth with SMC43 on 0.1X Difco plates. Two experiments were performed using different ratios of SMC43 and the soil solution inputs. For the MixnerA experiments, 500 µl of the

soil suspension were mixed with 500 µl of an SMC43 overnight culture in a sterile 2 ml tube. 50 µl of this mixture was spread on each of three 0.1x Difco plates with glass beads. For the MixnerB experiments, 100 µl of the soil suspension was mixed with 900 µl of a SMC43 overnight culture. As before, 50 µl of the mixture was spread onto each of three 0.1x Difco plates for culturing and DNA extraction (Figure 3.1E).

A final set of control experiments was conducted to identify microbes capable of directly binding to the applicator stick in the absence of SMC43 (referred to as 'Binder' experiments). Any bacteria that are able to do this would be unreliable MiPners if observed in such experiments, as we would be unable to distinguish whether they were observed due to their association with SMC43 or due to their natural binding capacity to the applicator stick. Sterile wooden applicators were set in a tube containing 1 ml of soil solution for 5 minutes, and afterwards were rinsed with 0.1X SSC and then dabbed onto a fresh Kimwipe to remove the excess liquid. Each of these applicators were used to streak onto 0.1X Difco plates for culturing and DNA extraction (Figure 3.1D).

Following all control experiments, the MiPner experiment entailed immersing the sterile wooden applicators first in a tube containing one ml of an overnight SMC43 culture (in 0.1X Difco) for 5 minutes. After 5 minutes, the applicators were rinsed with 0.1X SSC and then dabbed onto a fresh Kimwipe to remove excess liquid. The applicators were then immersed in a tube with one ml of soil solution. After 5 minutes, each applicator was individually rinsed with 0.1X SSC and then dabbed onto a fresh Kimwipe to remove excess liquid. Each of these applicators was used to streak onto 0.1X Difco plates for culturing and DNA extraction (Figure 3.1F).

Sequencing and Data Analysis of MiPner and Control Experiments

For sequencing library preparation, DNA concentration was measured fluorometrically and diluted to 2.5 ng/ul. Libraries were enzymatically fragmented and barcoded using the Plexwell plate-based library preparation system (SeqWell, California), following the manufacturer's protocol. Prepared libraries were sequenced on a NovaSeqX flow cell lane in PE150 mode (Illumina, San Diego, CA USA).

Raw sequencing reads were trimmed using fastp v0.23 (Chen et al. 2018). In the samples that correspond to the MiXner and MiPner steps, the bait was removed by mapping the reads to the genome assembly of SMC43 using BWA v0.7.17 (Li and Durbin 2009), filtering out with SAMtools v1.16.1 (Danecek et al. 2021) and removed with BBMap v38.98 (Bushnell 2014). All of these reads were classified using Kraken2 v2.1.3 (Lu et al. 2022; Wood, Lu, and Langmead 2019) with a paired-end setup. The reads were classified against the standard Kraken database which draws genomes from the NCBI RefSeq database (downloaded: March 27 2023). The relative abundances of genera were then estimated using Bracken 2.7 (Lu et al. 2022; 2017). Bracken reports were merged using kraken-tools v 1.2 (Lu et al. 2022), and archaeal, viral and human reads were removed from the bracken reports prior to summarizing genera and species abundances.

Sequencing and Genome Analysis of Isolated Colonies

DNA was extracted from isolated colonies with a protocol for high molecular weight DNA (Mayjonade et al. 2016). Libraries were prepared using the Rapid Barcoding kit (SQK-RBK004, Oxford Nanopore) and sequenced for 72 h using R9.4.1 flow cells (FLO-MIN106, Oxford Nanopore) on a GridION instrument. The genomes were assembled with Canu (Koren et al. 2017). The assemblies were taxonomically analyzed with TYGS (Meier-Kolthoff and Göker 2019).

Results

Isolation and characterization of a novel strain of S. marcescens *to use as bait.*

At the University of Georgia in Athens, GA (UGA), the course GENE4240L ("Experimental Microbiome Genetics") is designed to train students in the vagaries, certainties and uncertainties of discovery science. In 2021, students isolated myriad bacteria from a soil sample taken from an on-campus location at UGA. One of these bacteria produced red colonies and was isolated by rounds of streaking onto Difco plates, and then sequenced using Oxford Nanopore technology. The resultant sequence information indicated that this strain, named C4-3, was a S. marcescens that yielded an assembled chromosome of 5,092,593 bp with a 3,071 bp plasmid. Further annotation by the GENE4240L students who isolated this bacterium, Mary Norris and Molly Levine, indicated that it carried 4939 protein-encoding sequences in the chromosome and one in the plasmid (Figure 3.2).

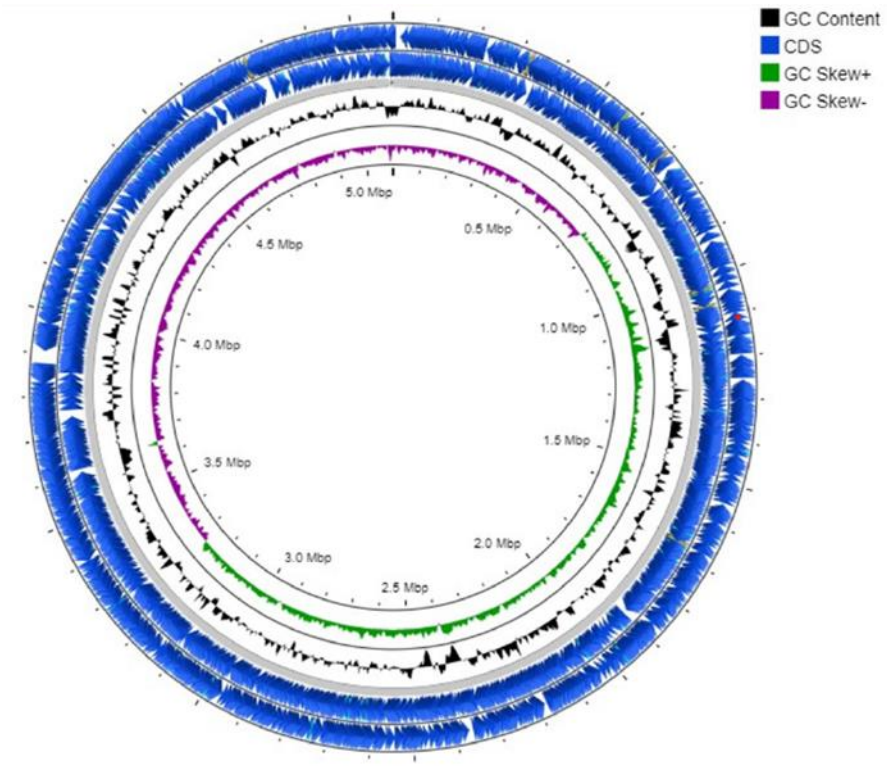

Figure 3.2 Genomic map and BRIG analysis of SMC43 featuring the circular map of the genome. Outer circle to inner circle (CDS, GC content, GC skew+, GC skew -, and GC).

Observed communities across the control experiments.

The bacterial community observed in the soil sample was highly diverse, containing a mean genera richness of 681.7 2.8 SE across the triplicate technical replicates. The dominant genera found within the soil were *Bradyrhizobium*, *Priestia* and *Streptomyces,* with a high abundance of low-frequency microbial groups (labelled as 'Other', Figure 3.3A). On the 0.1X Nutrient Agar plates, there were far fewer observed genera from culture (147.7 4.5SE), of which very few dominated the whole community (i.e., the 10 highest abundant genera combined constituted ~ 97% of all observed reads in the three replicates). The 0.1X Difco plates greatly enhanced growth of bacteria belonging to the genera *Enterobacter*, *Flavobacteria* and *Pseudomonas* at the expense of *Streptomyces*, *Priestia*, *Bradyrhizobium* and many other genera (Figure 3.3B).

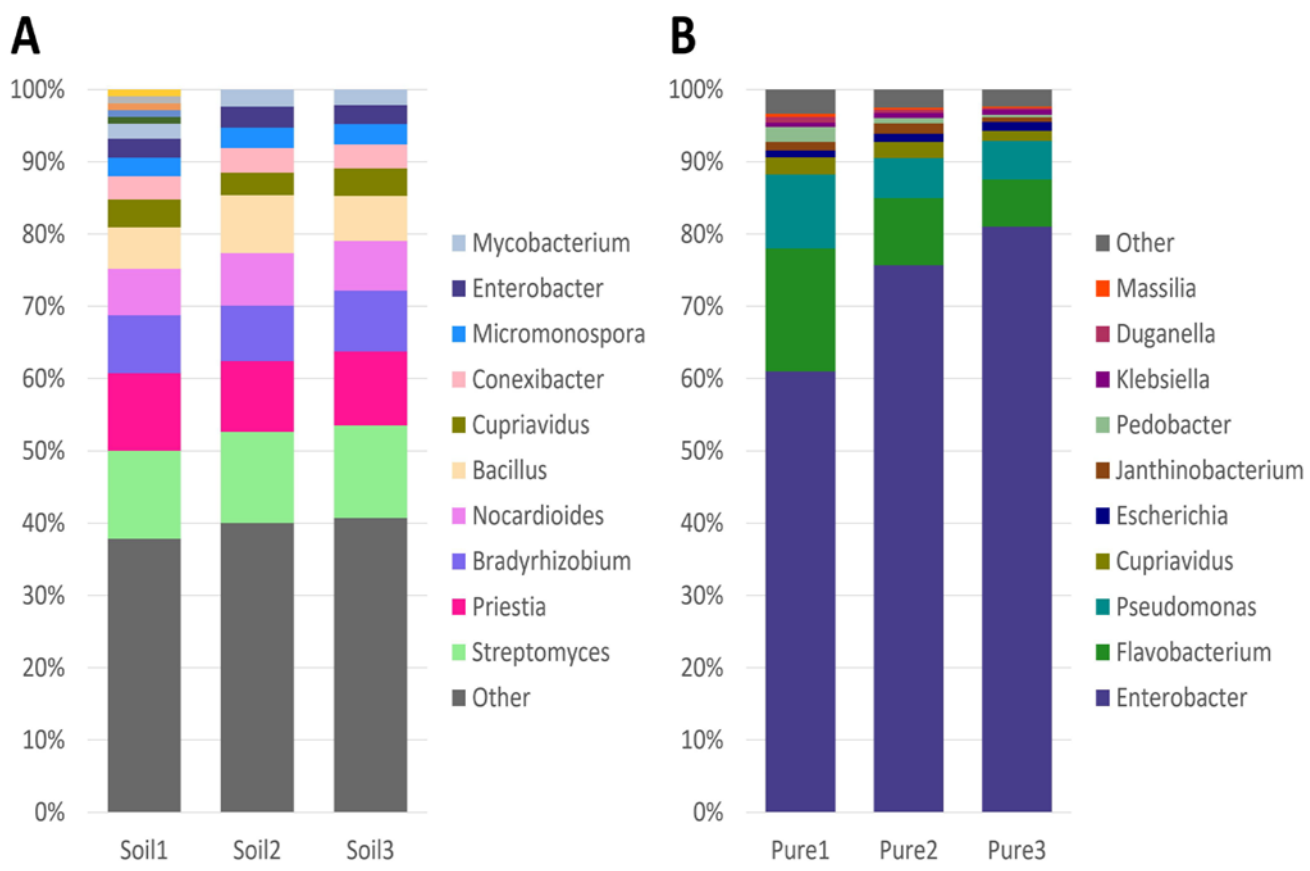

Figure 3.3 Taxa charts displaying the ten highest abundance genera of each treatment across from (A) the initial soil suspension (B) and the same suspension grown on 0.1X Difco plates for three days at room temperature. Genera are depicted in abundance order. Triplicate technical replicates are shown for each treatment.

Culturing the soil bacteria in the presence of SMC43 was expected to act as an additional selective force on the culturable bacteria. Figure 3.4 provides the results of mixing the soil solution with two different ratios of SMC43, and then growing this mixture for 3 days on 0.1X Difco plates. These results indicate that growth with SMC43 affected microbial diversity dramatically. Whereas growth on the plate without excessive SMC43 competition yielded an abundance of *Enterobacter* (Figure 3.3B), the SMC43-spiked plates enhanced the abundance of *Flavobacterium*, *Janthinobacterium* and *Pseudomonas* (Figure 3.4). Most Enterobacterial species were apparently

killed or otherwise growth-inhibited by SMC43. The dosage of SMC43 also made a difference, with *Pedobacter* and *Xanthomonas* doing better at a lower SMC43 dosage. We use the generic name "Mixner" to denote these microbes that survive growth with our bait microbe, SMC43. Thus, Mixners could be partners in interactions with SMC43 even if they do not bind under our study conditions.

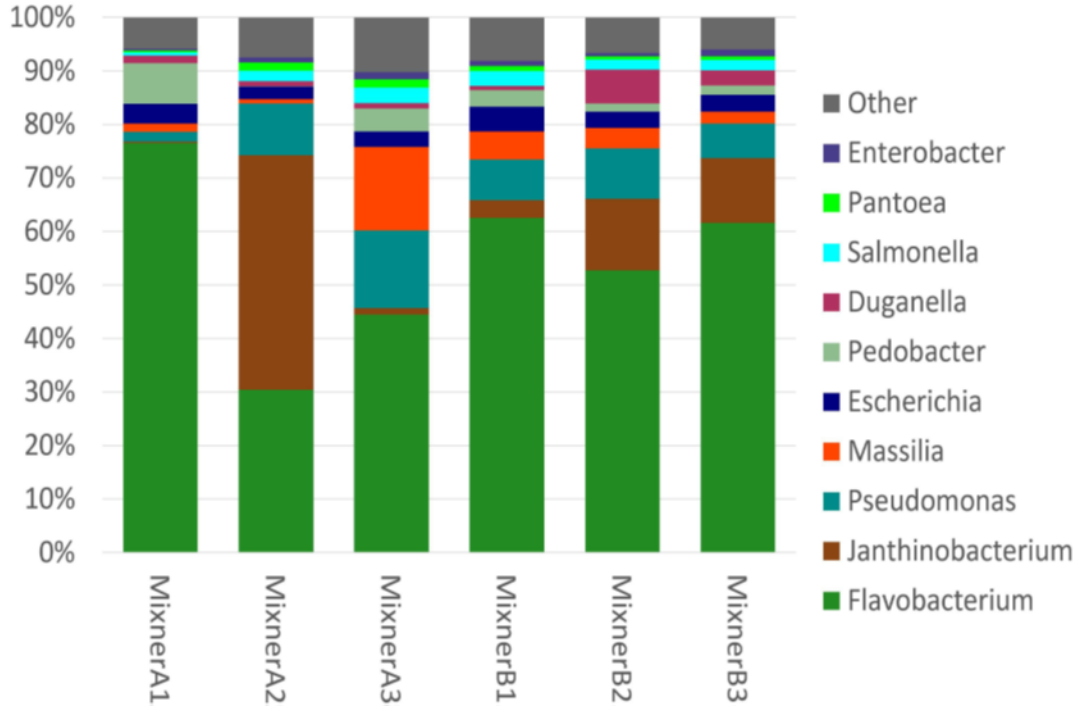

Figure 3.4 Taxa charts displaying the ten highest abundance genera from the MixNerA (10% soil suspension, 90% SMC43 overnight, left) and MixNerB (50% soil suspension, 50% SMC43 overnight, right) experimental cultures grown on 0.1X Difco plates for three days at room temperature. Genera are depicted in abundance order. For the MixNerA, an average of 83.3% of the reads were from SMC43 and in MixNerB, an average of 85.8% of the reads were from SMC43. SMC43 reads were removed so that the other bacteria on the plates could be revealed more clearly. Triplicate technical replicates are shown for each treatment.

Some of the apparent MiPners might be merely microbes that stuck directly to the applicator stick, and not to SMC43. Our stick-binding results indicated that members of the genera *Enterobacter*, *Chryseobacterium* and *Pseudomonas* were particularly avid binders to the applicator stick under these conditions from our studied soil solution (Figure 3.5). While most *Pseudomonas* can bind to the stick with varying degrees of success, there are four taxa that display marked increases in their relative abundance when grown with SMC43.

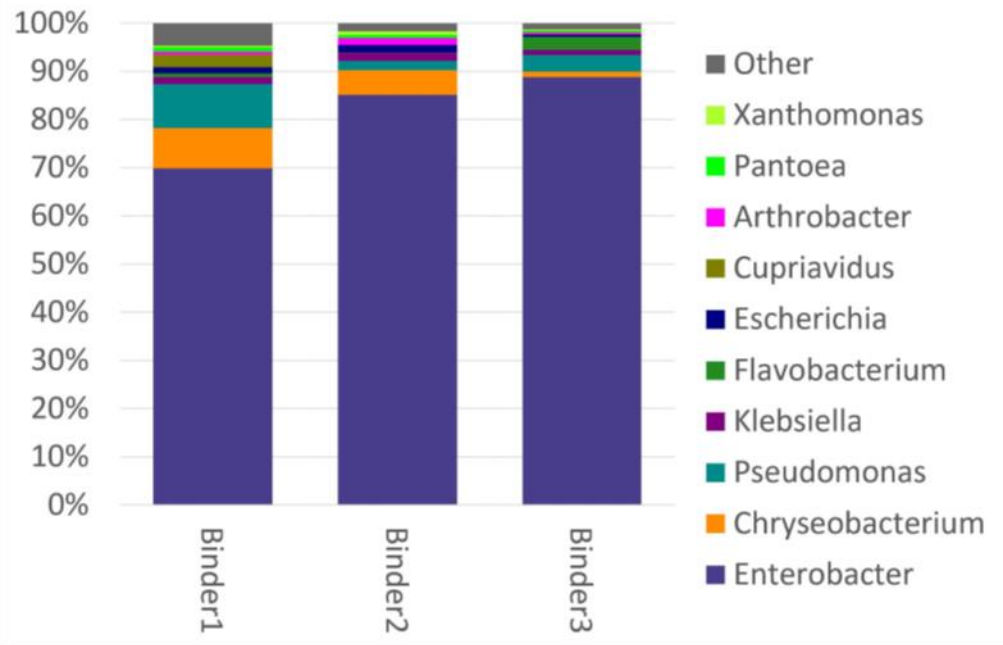

Figure 3.5 Taxa charts displaying the ten highest abundance genera that bound to the applicator stick in the absence of SMC43 when grown on 0.1X Difco plates for three days at room temperature. Genera are depicted in abundance order. Triplicate technical replicates are shown for each treatment.

MiPner identification and isolation

The observed community of MiPners varies across the triplicated technical replicates (Figure 3.6), a presumed outcome of the samples that were streaked on the Difco plates containing very few bacteria that passed all the requirements, especially binding to SMC43. In this case,

stochastic effects could play an influential role in inter-replicate variability because of low depth in the sampling. Regardless, at least two samples contained major representation of the genera *Pseudomonas*, *Sphingobium*, and *Caulobacter* (Figure 3.6). Despite its presence as the most abundant genus in the Mixner experiments (Figure 3.4), *Flavobacterium* was not among the ten highest abundant genera in these MiPner experiments, indicating a general inability to bind SMC43 under our study conditions.

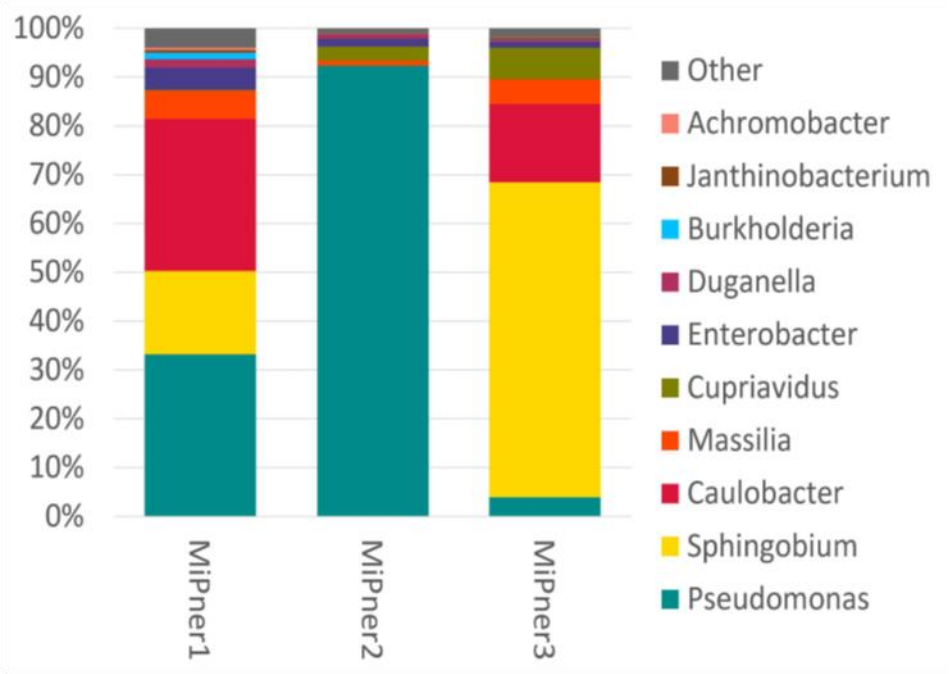

Figure 3.6 Taxa charts displaying the ten highest abundance genera observed from the MiPner experiments using the same SMC43 bait and different aliquots of the same soil solution when grown on 0.1X Difco plates for three days at room temperature. SMC43 reads were removed so that the other bacteria on the plates could be revealed more clearly. Triplicate technical replicates are shown for each treatment.

Genus-level enrichments across MiPner and control experiments

At the genus level, no *Enterobacter*, *Pseudomonas* or *Cupriavidus* could be trusted as a true MiPner isolate because of their strong affinity (see the "Binder Average" column, Table 4.1) for binding to the applicator sticks that we used, despite being found in high abundance in the MiPner samples. In contrast, *Sphingobium* and *Caulobacter* were not seen to bind to the applicator, and were rare in all steps, indicating at least 100-fold relative enrichment compared to the starting soil suspension.

Table 3.1 Mean relative abundances per experimental condition of the ten highest abundances putative MiPner genera identified from the MiPner experiments. Relative abundances are displayed across both the MiPner experiments and associated control experiments.

Ten Most Abundant MiPner Genera	**MiPner Average (%)**	**Binder Average (%)**	**Mixner A Average (%)**	**Mixner B Average (%)**	**Soil Solution on Plate Average (%)**	**Soil Solution Average (%)**
Pseudomonas	43.13	4.86	8.66	7.81	7.06	0.78
Sphingobium	27.19	Not present	1.08E-03	0.01	0.01	0.26
Caulobacter	15.76	Not present	4.24E-03	0.01	0.01	0.12
Massilia	3.96	0.16	5.99	3.76	0.32	0.28
Cupriavidus	3.17	0.91	0.29	1.13	1.99	3.65
Enterobacter	2.44	81.27	0.88	0.94	72.56	2.75
Duganella	0.96	0.10	1.13	3.32	0.49	0.06
Burkholderia	0.49	2.27E-03	0.03	0.08	0.04	0.57
Bradyrhizobium	0.38	3.76E-04	0.01	0.01	6.26E-04	8.17
Janthinobacterium	0.35	0.17	15.11	9.60	1.09	0.04

Species-level enrichments across MiPner and control experiments

At the species assignment-level, 33 individual taxa were identified belonging to the *Serratia* genus. *S. marcescens* was identified in expectedly high relative abundances across the experiments where SMC43 was added (MiPner: 64.24 % ± 6.35; MixNer A+B: 79.18 % ± 3.63,

Supplementary Table S1), and in low abundances within the binder (0.01 % ± 0.01) and pure (0.09 % ± 0.004) experiments. *S. marcescens* was not observed to be present in any soil samples. The presence of *S. marcescens* in binder and pure samples could therefore be due to either incredibly low-level cross-contamination of these samples during the experiments and / or sequence prep, or likely due to levels of *S. marcescens* (and other related species) in the soil extracts that was below the detection threshold of sequencing in these samples but not within other experiments where diversity was artificially lowered by experimental conditions. Due to the nature of Kraken2 / Bracken taxonomy assignment and abundance estimation, the majority of the remaining *Serratia* taxa sequence assignments are also likely derived from low-information DNA regions of the sequenced SMC43 isolate or related environmental taxa. Of the 32 *Serratia* taxa not directly attributed to *S. marcescens*, only four were found in experiments where SMC43 was not added, and when they were recovered in these samples, they were in incredibly low abundance (< 0.01% mean relative abundance in any experiment group). This demonstrates that for mixed bacterial communities, the interpretation of species-level assignments based on Kraken2 / Bracken requires some caution.

All *Serratia*-related taxa were removed for subsequent analyses, so relative abundances of the remaining taxa were calculated without *Serratia*. The top 100 most abundant taxa across the MiPner experiments were considered to determine which individual taxa could be regarded as putative MiPners based on their presence in both the MiPner experiment and 'Binder' control experiments. 18 taxa were identified with > 1% relative abundance in at least one MiPner experiment sample, of which 6 were identified as putative MiPners, and 12 could not be assigned MiPner status due to their presence in the binder control experiments. Putative MiPners include *Sphingobium yanoikuyae*, *Caulobacter segnis*, *Sphingobium sp.* PAMC28499, *Sphingobium sp.*

LF-16, *Caulobacter vibrioides* and *Burkholderia thailandensis*. Of the 12 taxa which could not be confidently assigned MiPner status with high abundances, there were 6 *Pseudomonas*, 3 *Masilia*, 1 *Cupravidus*, 1 *Duganella*, and 1 *Enterobacter* assigned taxa.

21 additional putative MiPners were identified at a low abundance (< 1% relative abundance in at least one MiPner experiment sample). These included several taxa that were not considered when examining genus level associations including *Bordella parapertussis*, *Diaphorobacter polyhydroxybutyrativorans*, *Nordella sp.* HKS 07, *Pectobacterium carotovorum*, *Lautropia mirabilis* and *Yersinia Pestis*. Additionally, there were 5 *Caulobacter*, 4 *Sphingobium*, 2 *Bradyrhizobium*, 2 *Masilia* and 2 *Pseudomonas* taxa identified as putative MiPners. The detection of Pseudomonads as putative MiPners in contrast to our genus-level assessment shows that there may be some utility to considering a species level definition. As with all low-abundance taxa however, these two identified *Pseudomonas* MiPners are unlikely to be readily detectable through onward culturing, and when including the high abundance (> 1%) Pseudomonads detected represent only two out of 36 observed taxa in this genus. Low capacity for species resolution as demonstrated by our *Serratia* analysis also calls into question how definitive this approach can be in comparison to genus level assessments without onward culturing.

MiPner genome sequences and phylogenetic assessment.

Our results demonstrate that the MiPner analysis excluded most microbes from the soil solution while selecting a tiny subset that could both bind SMC43 and grow with it under the conditions investigated. As can be seen (Figure 3.6), *Pseudomonas*, *Sphingobium*, and *Caulobacter* were the most abundant genera represented in this MiPner experiment.

In order to characterize particular MiPners, we picked several of the colonies that were growing on top of the SMC43 streak, for additional streaking on 0.1X Difco plates. The plates

from such colony picking indicated a segregation of red and tan colonies. For two of these tan colonies, we sequenced their genomes as described for the sequencing of SMC43. The sequences indicated that one was a strain of *Pseudomonas monteilii* with a 6.3 Mb genome and the other was a strain of *Enterobacter asburiae* with a 3.99 Mb genome. Although *P. monteilii* was not one of the *Pseudomonas* species that bound strongly to the applicator stick, *E. asburiae* was a strong stick binder (Figure 4), so it seems likely that the *E. asburiae* that we isolated was not actually a true MiPner that bound strongly to SMC43.

Additional MiPner experiments from different soil sources and different times of year.

Because soil microbiomes change dramatically over time (Habekost et al. 2008; Landesman, Freedman, and Nelson 2019) (and depending on the soil type and plant root types/abundances (Bulgarelli et al. 2013; Chaluvadi and Bennetzen 2018; Richter-Heitmann et al. 2016), we performed two additional MiPner experiments with soil collections from different sites, but using SMC43 as bait. Our hypothesis was that the MiPners that bound to SMC43 would be different in these different soil samples. The same technique was pursued as in the first experiment, and 24 colonies on the SMC43 streak were picked and streaked onto 0.1 Difco plates. Of these, 22 picked MiPners yielded red and tan colonies. Two of these tan colonies, from different initial soil collections, were purified and sequenced. One was found to be an ecotype of Stenotrophomonas maltophilia with a 4.63 Mb genome and the other was found to be a novel *Mitsuaria* species, with a 3.62 Mb genome, that was most closely related to *Mitsuaria chitinivorans*.

The other two picked MiPners only yielded red solo colonies, but continued to show mixed red/tan bacterial growth in the denser streaking that did not separate out solo colonies. We attempted to sequence one of these, called 3B1D, presumably a mixture of SMC43 and the MiPner, using Oxford Nanopore Technologies platforms. This DNA sequence analysis of 3B1D indicated

that there was both one Mitsuaria and one SMC43 genomes present, suggesting that this *Mitsuaria* could not grow on this plate type, under our conditions, in the absence of its SMC43 partner. This *Mitsuaria* appears to be a previously uncharacterized species that is most closely related to *Mitsuaria nodulii*.

Discussion

One of the most challenging problems in the study of microbe-microbe interactions in the real world is that we neither understand the particular micro-environments in which these interactions occur nor have we even a faint idea of the dynamics and depths of involvement of different biological participants. Attempts to create synthetic communities are praiseworthy but are not proven to actually replicate interactions that occur in nature (McClure et al. 2020; Niu et al. 2017; Yin, Hagerty, and Paulitz 2022; Zengler et al. 2019). We decided to take a different starting point, an apparent interaction, and create a system that works from that beginning. Obviously, once a two-component community is generated and investigated, adding additional components (established, for instance, by seeing what uniquely binds to a pair of interacting MiPners) will be feasible (Davis, Joseph, and Janssen 2005). The challenges of growing soil bacteria. As has been heavily documented (Ernebjerg and Kishony 2012; Lloyd et al. 2018; Rappé and Giovannoni 2003; Shade et al. 2012; Stewart 2012), most soil bacteria have been (so far) recalcitrant to growth on plates, even though many plate types and growth conditions have been tested. Our experiments growing soil bacteria on 0.1X Difco plates at room temperature under aerobic conditions indicated a great depletion of acidobacteria and actinobacteria, and a great over-representation of proteobacteria, as has been frequently shown by others (Rappé and Giovannoni 2003). We expect that different plating conditions would yield different enrichment/depletion patterns (Davis, Joseph, and Janssen 2005; Pham and Kim 2016; 2012). Why these microbes are

recalcitrant to culturing is not known, but it is expected to be associated with an unknown necessary component or components of their microenvironments (Pham and Kim 2012). Perhaps one of these unknown components is a microbial partner (Burmølle et al. 2009), as manifested in our SMC43-*Mitsuaria*-3B1D result. Syntrophic interactions are not uncommon in microbial networks, of which this interdependence and need for co-culture may be an example (Dillon and Dillon 2004). We have seen other such "cannot grow without the bait" examples from MiPner experiments with other bait species (unpublished), so it is possible that MiPner technology may be one general tool for future isolation of such recalcitrant microbes. Of course, our SMC43-associated *Mitsuaria* may be able to grow on some plate types if we pursued a full round of investigations. Many *Mitsuaria* grow on several different plate types, as we have seen in our lab, including 0.1X Difco plates (Fan et al. 2018) but we believe it is likely that many such future "bait-requiring" microbe isolations will be of species that have an absolute partner requirement. Regardless, the SMC43-*Mitsuaria*-3B1D interaction on 0.1X Nutrient Agar plates indicates a pairwise interaction that is obligate for this *Mitsuaria*'s growth, and future studies showing what SMC43 provides in this relationship will be of great interest.

MiPner specificity.

The very different and taxonomically limited set of MiPner microbes identified, compared to our starting soil and to other selective steps (e.g., plate type) in the technique, indicates that the microbe-microbe binding is highly selective and robust enough to avoid removal by a simple rinsing step. Moreover, this binding requires only a few minutes to generate this specificity and durability. Preliminary studies in our lab using different bait species on the same soil suspension (unpublished results) have suggested that each bait generates a different set of MiPners that are enormously enriched at the genus level. Once pursued, we expect that observed species-level

binding specificities and enrichments will be even more dramatic. Hence, there is an unlimited potential for using MiPner technology to find potential interacting partners with most other microbes, and this should extend beyond just bacteria.

Isolation of colonies growing on the bait streak is not likely to only yield MiPners, as shown with our *E. asburiae* result. Anything that binds the applicator stick found in the study does not need to bind the bait, although it is required to grow with the bait on the plate type utilized. For this study, we would be very confident of the MiPner status of any *Sphingobium* or *Caulobacter* isolated, which could be further confirmed by reciprocal binding studies. The full set of potential bait partners.

The full set of potential bait partners

There is no reason to believe that any bacterium interacts with the same set of microbial partners in all environments. Our investigations of different soils with SMC43 indicated very different final MiPner outcomes, dependent on soil source. The two *Mitsuaria* that we found in a subsequent experiment to the one described in detail here, represent a genus that was fully absent from all of our sequencing in the first experiment. Hence, discovery of the full set of SMC43 MiPners that may be real-life microbial partners would be best pursued with a number of different soil sources. And this will be equally true for any other microbe used as bait. MiXner results.

Mixner results

We call the community DNA sequence results of growing one bait microbe with a soil suspension of microbes to be the outcome of a Mixed preMiPner, or Mixner, experiment. Our Mixner outcomes indicate variable survival patterns of the soil-solution microbes depending upon the dose of the bait microbe. Of course, SMC43 may provide a severe example of this phenomenon

because of its production of prodigiosin, a potent anti-microbia (Lapenda et al. 2015). However, many bacteria produce antimicrobials, so we predict this ratio-dependence result to be generally true with any bait microbe or any mixed microbe suspension. Perhaps at lower bait dosages, other microbes have a greater opportunity to build communities that will resist any negative (or positive) contributions from the bait microbe.

MiPner enrichment.

Several steps of enrichment led to the isolated microbes that were dubbed MiPners. The selection of a liquid suspension, rather than total soil, as the initial soil microbe source was one such enrichment/depletion step. As noted, growth on a plate and survival of exposure to SMC43 were other selection steps, each with unique outcomes. The primary goal of this technology, and thus the most interesting enrichment for us, is to find paired candidates for a specific SMC43-MiPner interaction. Starting with these functional components of a durable and highly-specific binding, study of these two-species interactions can proceed in a wealth of directions. Characterization of each of these pairs of interactions are warranted by such techniques as annotation for gene-enrichment by the selection process, optical studies of the physical interaction, forward genetic searches for genes that decrease or increase the partnership, reverse genetics of genes likely to be involved in the binding and other interactions, transcriptomic/proteomic/metabolomic analysis of inductions/repressions by the interactions, and many others too numerous to list. All of these are beyond the scope of our current investigation, but not of the experiments we are currently pursuing.

It should be noted that none of the controls that we pursued in this study would be necessary to pull out MiPners that bound to the bait. Just the simple bait binding to an applicator, followed by the second immersion and plating, would be sufficient to find a candidate partner. However, the

various controls were of great value in determining the likelihood that the identified microbes truly were MiPners, especially in distinguishing microbes that bound to the applicator stick without bait involvement. Full confirmation of a MiPner would be best pursued by subsequent studies, especially by using the MiPner as bait to see if it pulls out its partner that was the initial bait.

Generality of the MiPner strategy

As should be clear, there is absolutely nothing about the MiPner strategy that is limited to any specific bait microbe or to any specific microbial community. The animal gut (Dillon and Dillon 2004; Gill et al. 2006; Hitch et al. 2022) or bodies of water (Diao et al. 2017; Venter et al. 2004) and such fascinating microbial worlds as permafrost (Wu et al. 2022), the digestive fluids of pitcher plants (Zhang, Chaluvadi, and Bennetzen 2020a; 2020b) or waste tailings (Yagi et al. 2009) will be equally accessible to this technology. There is potential for viruses, fungi, protists, tiny invertebrates or archaea to be used as baits or followed as MiPners. Moreover, it is difficult to appropriately convey the amazing speed, simplicity, robustness and low cost of this approach. We hope that many laboratories will join us in MiPner experimentation, so that we can begin to assemble a microbial interaction atlas, starting with two species at a time.

Chapter 4

GENOME-ENABLED BACTERIAL SPECIES DISCOVERY

Introduction

Soil is the most complex and diverse microbial habitats on Earth, hosting a vast array of microorganisms from all three domains of life—Bacteria, Archaea, and Eukarya (Sokol et al. 2022; Vos et al. 2013). The soil microbiome is responsible for essential ecosystem functions and services, such as plant fitness and productivity, nutrient cycling, organic matter decomposition, pollutant degradation, and pathogen control (Delgado-Baquerizo et al. 2020). Despite their significance, much of the microbial diversity within soil remains poorly understood, even for some of the most abundant and well-studied bacterial and fungal groups (Vitorino and Bessa 2018). This knowledge gap is largely due to the inherent complexity of soil ecosystems and the limitations of traditional culture-based methods, which typically isolate less than 1% of soil microbial diversity (Young and Crawford 2004), and the lack of a clear species framework for microorganisms.

Recent advances in genome sequencing technologies, combined with innovations in bioinformatics, have revolutionized the discovery of new bacterial species in soil. The decline in the cost of whole genome sequencing, the enrichment of genomic databases, and the increase in computational power have given rise to genome-based taxonomy methods (Meier-Kolthoff and Göker 2019). These methods analyze entire genome sequences or significant portions of them to determine relationships between bacterial species and classify them with high accuracy and resolution.

Historically, microbial taxonomy was based on phenotypic characteristics such as cell morphology, metabolic capabilities, and growth conditions (Cohan 2002). However, these characteristics are subject to direct selection, and thus often exhibit convergent evolution, so they often lacked the resolution needed to precisely distinguish species (Cohan 2002). In the 1960s DNA-DNA Hybridization (DDH) was developed to distinguish new species and was long considered the gold standard for determining genome similarity in bacterial taxonomy (Wayne et al. 1987). However, DDH is an indirect measure, known for being error-prone, labor-intensive, technically challenging, could only be done in a few selected laboratories (J. L. Johnson and Whitman 2014; Lee et al. 2016) and generally could only investigate two organisms per comparative hybridization. With the advent of whole-genome sequencing, several overall genome relatedness indices (OGRIs) have been developed to replace DDH (Lee et al. 2016). These indices allow for direct genome sequence comparison, offering more objective, reproducible, and efficient alternatives to traditional methods (Lee et al. 2016).

Among these, Average Nucleotide Identity (ANI) has become the most widely used (Beaz-Hidalgo et al. 2015; Lee et al. 2016; Rosselló-Móra and Amann 2015). ANI is often referred to as a digital version of DDH, as it was initially designed to mimic the experimental process of DDH (Goris et al. 2007; Konstantinidis, Ramette, and Tiedje 2006; Lee et al. 2016). ANI was initially developed to replicate the experimental process of DNA-DNA hybridization (DDH) and is therefore sometimes referred to as the digital equivalent of DDH (Lee et al. 2016). It is calculated by comparing the genome sequences of two strains (the query and subject). The process begins by breaking the genome sequence of the query strain into fragments, each about 1020 base pairs long. Next, these fragments are aligned against the entire genome of the subject strain using NCBI's BLASTn tool. During this step, BLASTn calculates the nucleotide identity between the query

fragments and the subject genome. The ANI is the average of these individual identity values. ANI provides a more accurate and standardized approach by defining species boundaries based on genetic similarity, typically using a threshold of at least 95-96% identity to demarcate species. This approach has led to the reclassification of many microbial species and the discovery of entirely new ones that were previously misidentified or overlooked.

Despite their microscopic size, bacteria play crucial roles in maintaining the earth's biosphere. Yet, they remain one of the largest unexplored reservoirs of biodiversity. A major challenge in understanding this diversity is the lack of a universally accepted species concept for bacteria (Ogunseitan 2005; Bobay 2020; Cohan 2002). In contemporary bacterial and archaeal taxonomy, new species are identified through a polyphasic approach, which integrates multiple dimensions of an organism, including phenotypic, genotypic, and chemotaxonomic traits (Cohan 2002; Konstantinidis, Ramette, and Tiedje 2006). Nevertheless, the genome remains the most comprehensive source of taxonomic information. Because most soil bacteria are difficult to culture, and due to the replicability of computational analyses, genome-enabled species discovery holds promise for deepening our understanding of the vast microbial diversity hidden within soil.

Methods

Soil sources and bacterial isolation

Samples were isolated from diverse sources. Janthinobacterium sp. 2021 was isolated from soil collected in January 2021 outside the Davison Life Sciences Complex (GPS coordinates: 33°56'35.8'' N, 83°22'23.1'' W). *Janthinobacterium sp. 2022* and *Pseudomonas sp. 3-2* were isolated from soil collected from a sorghum field in January 2022 (GPS coordinates: 33°53'31.7" N, 083°25'31.1" W). Bacteria isolated using the MiPner technique were obtained from soil from the same sorghum field collected in July of 2022. A portion of the soil collected for the MiPner

experiment was stored (and thus naturally desiccated) at 25 °C in an open container for 3 months. Root endophytic bacteria were obtained from a red mangrove *(Rhizophora mangle)* tree that had fine roots harvested on Saint George Island in May 2019 (29°40'16.4" N, 84°51'09.1"W).

Soil samples were processed by mixing the soil with 0.1X phosphate buffer saline (PBS) and serially diluted to 10^{-1}, 10^{-2}, 10-3, and 10^{-4}. 100 microliters of each of the dilutions and the undiluted soil suspension were spread onto either Nutrient Agar (Difco) or Soil Extract (HiMedia) plates. Plates were incubated for two days at room temperature. Colonies of interest were purified through several rounds of streaking and re-culturing on Nutrient Agar (Difco) plates. Individual colonies were then picked into nutrient broth for growth and eventual storage at -80°C in 25% glycerol.

MiPner bacteria were isolated by making a soil solution of 15 g of soil mixed with 300 ml 0.1 X saline-sodium citrate (SSC) buffer (0.15M Sodium Chloride and 15mM Sodium Citrate, pH 7.0). The mixture was shaken at 250 rpm for 10 minutes and left for a further 10 minutes for most particulate matter to settle at the bottom of the container. The upper 25% of this suspension was decanted into a sterile 500 ml container. The MiPner bacteria were isolated as described in Chapter 3 of this dissertation.

For the mangrove-associated isolates, fine root fragments from a young red mangrove were rinsed and ground in 1X PBS, and the supernatant was spread onto Eosin Methylene Blue (EMB) (HiMedia) and Pikovskaya (HiMedia) media plates. Colonies were picked, serially streaked onto the same media three times, and then individual colonies were picked into nutrient broth for growth and eventual storage at -80°C in 25% glycerol.

For the sorghum rhizoplane isolate, seeds of sorghum *(Sorghum bicolor)* Grassl were surface sterilized by placing them in a 10% bleach solution for 30 minutes, washed with sterile

water 3 times, soaked overnight in 1% Captan, and transferred to a field plot on the campus of the University of Georgia (33° 55’ 44.9’’ N, 83° 21’ 46.1’’ W). After three weeks, the seedlings were collected and moderately washed with 0.1X saline-sodium citrate (SSC) buffer. The seedlings were carefully placed on 150 mm x 15 mm Petri dishes, containing Soil Extract (HiMedia) media. Apparent single colonies were picked and serially streaked onto the same media three times, and then individual colonies were picked into nutrient broth for growth and eventual storage at -80°C in 25% glycerol.

DNA Extraction and Sequencing

High molecular weight DNA was extracted using the protocol of (Mayjonade et al. 2016). DNA libraries were prepared for sequencing using the Rapid Barcoding Kit following the manufacturer’s instructions (SQK-RBK004, Oxford Nanopore Technologies, Oxford UK). The libraries were sequenced for 72 hours using a R9.4.1 flow cell (FLO-MIN106, Oxford Nanopore Technologies) on the GridION instrument.

Bioinformatic Analyses

Raw reads were corrected then trimmed and assembled using Canu (Koren et al. 2017). The assemblies were submitted to TYGS (Meier-Kolthoff and Göker 2019) for a genome based taxonomic analysis. If TYGS suggested that an isolate was a new species the type genomes of the nine most closely related species were downloaded and used to calculate the average nucleotide identity using OrthoANI (Lee et al. 2016). The isolates and their most closely related genomes were annotated using Prokka (Seemann 2014), and their gene contents were compared using Roary (Page et al. 2015).

Results

We identified 11 new bacterial species based on ANI values below 95%, spanning 7 different genera (Table 4.1). The genus *Pseudomonas* had the most newly identified species, with three representatives, followed by *Janthinobacterium* with two. The percentage of shared genes ranged between 12.97% between *Mucilaginibacter sp.* DS21 and Mucilaginibacter celer; and 62.93% between *Pseudomonas sp.* 3-2 and *Pseudomonas aphyarum* (Table 4.1).

Table 4.1 Taxonomic information on the isolates, and shared genes.

Isolate	**Most Closely Related Species**	**ANI**	**Shared Genes**	**Unshared Genes**
Janthinobacterium sp. 2021	*Janthinobacterium rivuli*	92.51	2,748 (33.30%)	5,504 (66.70%)
Janthinobacterium sp. 2022	*Janthinobacterium rivuli*	92.09	2,988 (34.16%)	5,758 (65.84%)
Mucilaginibacter sp. DS21	*Mucilaginibacter celer*	83.78	1,608 (12.97%)	10,790 (87.03%)
Burkholderia sp. 1B3	*Burkholderia orbicola*	94.45	4,329 (44.74%)	5,346 (55.26%)
Chryseobacterium sp. 1B3	*Chryseobacterium gleum*	92.81	2,741 (36.28%)	4,815 (63.72%)
Pseudomonas sp. 2A5	*Pseudomonas grandcourensis*	92.45	2,547 (30.05%)	5,928 (69.95%)
Variovorax sp. 2A5	*Variovorax guangxiensis*	93.59	3695 (39.06%)	5,765 (60.94%)
Pseudomonas sp. 3-2	*Pseudomonas aphyarum*	94.85	4,457 (62.93%)	2,626 (37.07%)
Mitsuaria sp. S21	*Mitsuaria chitinivorans*	92.54	2,819 (37.21%)	4,756 (62.79%)
Pseudomonas sp. S32	*Pseudomonas putida*	89.81	3,007 (33.66%)	5,926 (66.34%)
Pseudomonas sp. SR04	*Pseudomonas beijingensis*	93.4	3,798 (46.92%)	4,296 (53.08%)

These bacterial isolates were obtained from diverse environments, including soils and mangrove roots, utilizing various isolation techniques (Table 4.2). Soil plating was the most used technique. This method yielded most of the *Janthinobacterium* and *Pseudomonas* isolates (Table 4.2).

Table 4.2 Sources and isolation techniques for bacterial isolates.

Isolate	Source	Isolation Technique
Janthinobacterium sp. 2021	Soil	Soil plating
Janthinobacterium sp. 2022	Sorghum field soil	Soil plating
Mucilaginibacter sp. DS21	Desiccated sorghum field soil	Soil plating
Burkholderia sp. 1B3	Sorghum field soil	MiPner
Chryseobacterium sp. 1B3	Sorghum field soil	MiPner
Pseudomonas sp. 2A5	Sorghum field soil	MiPner
Variovorax sp. 2A5	Sorghum field soil	MiPner
Pseudomonas sp. 3-2	Sorghum field soil	Soil plating
Mitsuaria sp. S21	Mangrove fine roots	Root grinding
Pseudomonas sp. S32	Mangrove fine roots	Root grinding
Pseudomonas sp. SR04	Sorghum rhizoplane	Root plating

Most assemblies were complete, except for some of the MiPner sequences (Table 4.3). Genome sizes range from around 4.99 million base pairs (bp) in *Chryseobacterium* sp. 1B3 to over 8 million bp in *Burkholderia sp.* 1B3. Most genomes fall between 5.77 and 7.76 million bp. Both *Janthinobacterium sp.* 2021 and *Pseudomonas sp.* S32 had plasmids.

Table 4.3 Genomic characteristics of newly discovered species.

Isolate	Genome Size	Contigs	GC
Janthinobacterium sp. 2021	6,164,718	2	63.1
Janthinobacterium sp. 2022	6,693,321	1	63.1
Mucilaginibacter sp. DS21	7,764,601	1	43.02
Burkholderia sp. 1B3	8,030,667	3	66.72
Chryseobacterium sp. 1B3	4,995,114	1	37.47
Pseudomonas sp. 2A5	6,042,350	31	59.61
Variovorax sp. 2A5	5,965,519	2	67.7
Pseudomonas sp. 3-2	6,263,213	17	60.8
Pseudomonas sp. S32	5,772,064	2	61.6
Mitsuaria sp. S21	6,067,743	1	69.8
Pseudomonas sp. SR04	7,058,539	7	61.22

Soil Janthinobacterium

Janthinobacterium sp. 2021 has a genome size of 6,164,718 base pairs consisting of one chromosome and one plasmid. The GC content is 63.1%. The N50 value is 6,145,446, while the L50 is 1 (Table 4.3). *Janthinobacterium sp.* 2021 has 5,427 protein coding genes and 98 RNA genes.

Janthinobacterium sp. 2022 has a genome size of 6,693,321 base pairs. It is represented by a single contig. Like *Janthinobacterium sp.* 2021, this isolate has a GC content of 63.1% (Table 4.3). *Janthinobacterium sp.* 2022 has 6161 protein coding genes and 101 RNA genes.

Both *Janthinobacterium sp.* 2021 and *Janthinobacterium sp.* 2022 have Average Nucleotide Identity (ANI) scores <95 to all other *Janthinobacterium* genomes. They are both closely related because they have the highest ANI to each other, but, still, this is below 95, indicating these are distinct new species (Figure 4.1). The highest ANI for both was to *Janthinobacterium rivuli*, originally isolated from a freshwater stream in China (H. Lu et al. 2020). Genes shared between *Janthinobacterium sp.* 2021 and *Janthinobacterium rivuli* were 2,748 (33.30%), while 5504 (66.70%) were unshared (Table 4.1). *Janthinobacterium sp.* 2021 and *Janthinobacterium rivuli* shared 2,988 (34.16%) genes, while 5,758 (65.84%) were unshared (Table 4.1).

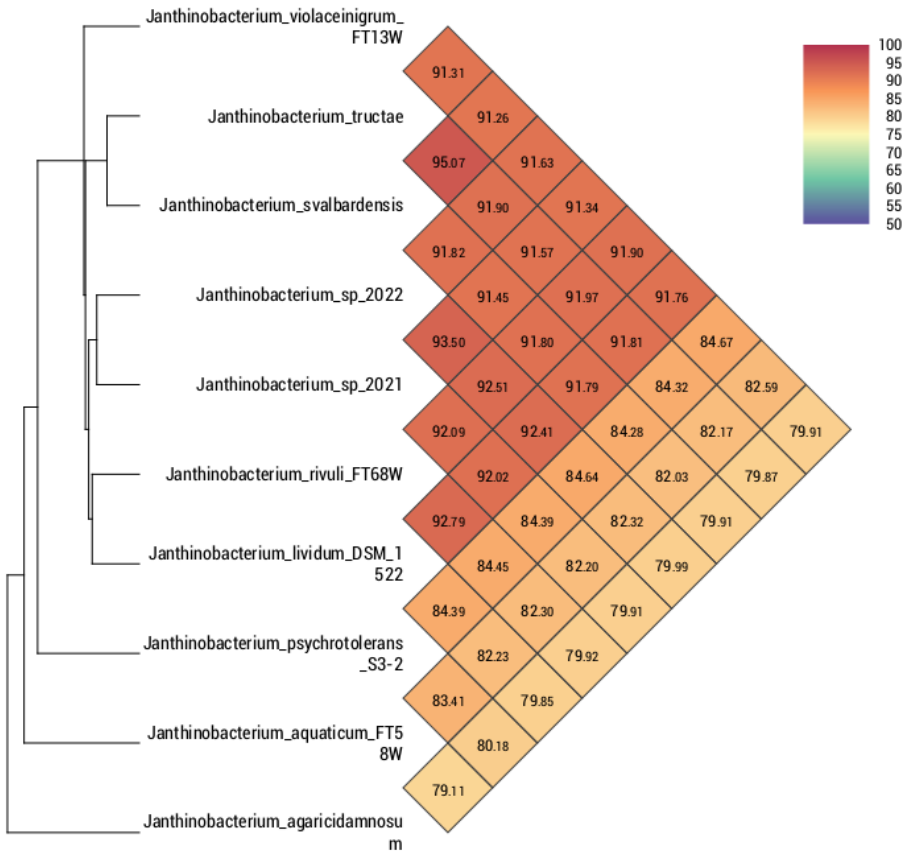

Figure 4.1 Average Nucleotide Identities of *Janthinobacterium sp.* 2021 and *Janthinobacterium sp.* 2021.

Desiccated Soil

Mucilaginibacter sp. DS21 has a genome size of 7,764,601 base pairs in one single chromosome and the GC content is 43.02% (Table 4.3). There were 8,036 protein coding genes and 63 RNA genes identified in *Mucilaginibacter sp.* DS21.

Mucilaginibacter sp. DS21 had the highest ANI (83.78%) to *Mucilaginibacter celer* (Figure 4.2), which was originally isolated from a lake in South Korea (Kim, Shin, and Yi 2020). The two species *Mucilaginibacter sp.* DS21 and *Mucilaginibacter celer* share 1,608 (12.97%) genes, while 10,790 (87.03%) were not shared (Table 4.1).

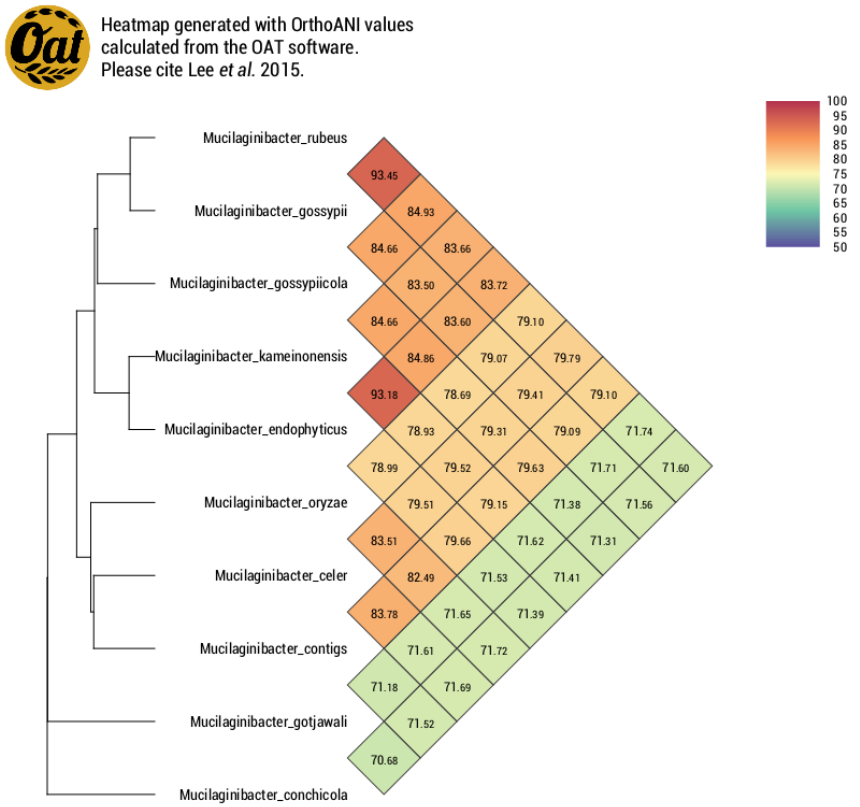

Figure 4.2 Average Nucleotide Identities of *Mucilaginibacter sp.* DS21

MiPners

The first MiPner experiments were done using *Janthinobacterium sp.* 2021 and *Janthinobacterium sp.* 2022 as the bait, during this time there were none of the controls described in the previous chapter. These experiments led to the isolation of members of the genera *Burkholderia sp.*, *Chryseobacterium sp.*, *Pseudomonas sp.*, and *Variovorax sp.*

Burkholderia sp. 1B3 was isolated using *Janthinobacterium sp.* 2021 as bait. It has a genome size of 8,030,667 base pairs and is composed of 3 contigs. The GC content is 66.72% (Table 4.3). There were 7,335 protein-coding sequences and 77 RNA genes. *Burkholderia sp.* 1B3 had its highest ANI (94.45%.) to *Burkholderia orbicola*, which was originally isolated from the tomato rhizosphere (Morales-Ruíz et al. 2022). Both species share 4,329 genes (44.74%), while 5346 (55.26%) were not shared (Table 4.1).

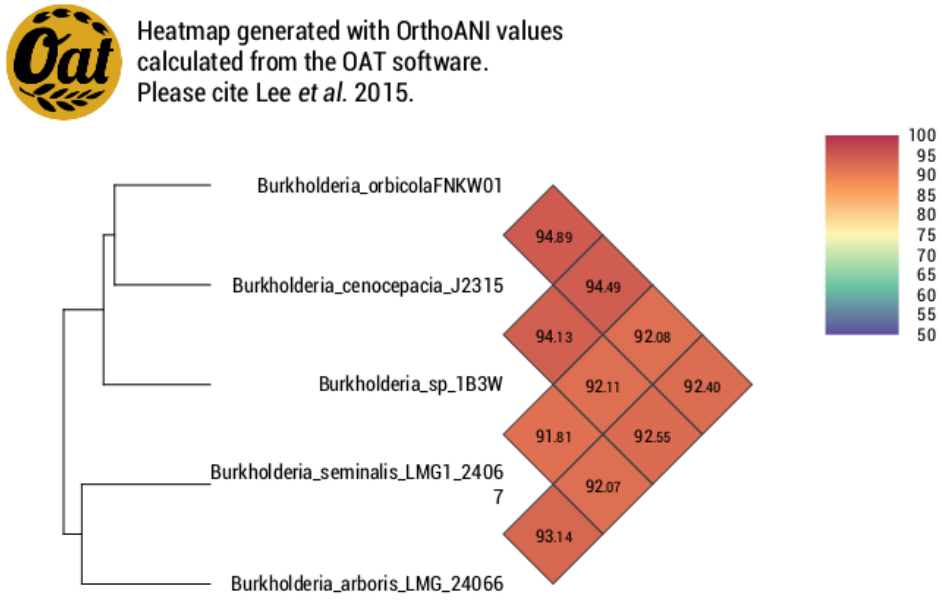

Figure 4.3 Average Nucleotide Identities of *Burkholderia sp.* 1B3.

Chryseobacterium sp. 1B3 was isolated using *Janthinobacterium sp.* 2021 as bait. It has a genome size of 4,995,114 base pairs in one single contig and the GC content is 37.47% (Table 4.3). There were 5,158 protein-coding sequences and 89 RNA genes predicted. *Chryseobacterium sp. 1B3* had its highest ANI score (92.81%) to *Chryseobacterium gleum*, which was originally described as *Flavobacterium gleum* and isolated from clinical samples (Holmes et al. 1984). There were 2741 (36.28%) genes shared between *Chryseobacterium sp. 1B3* and *Chryseobacterium gleum*, while 4815 (63.72%) were not shared.

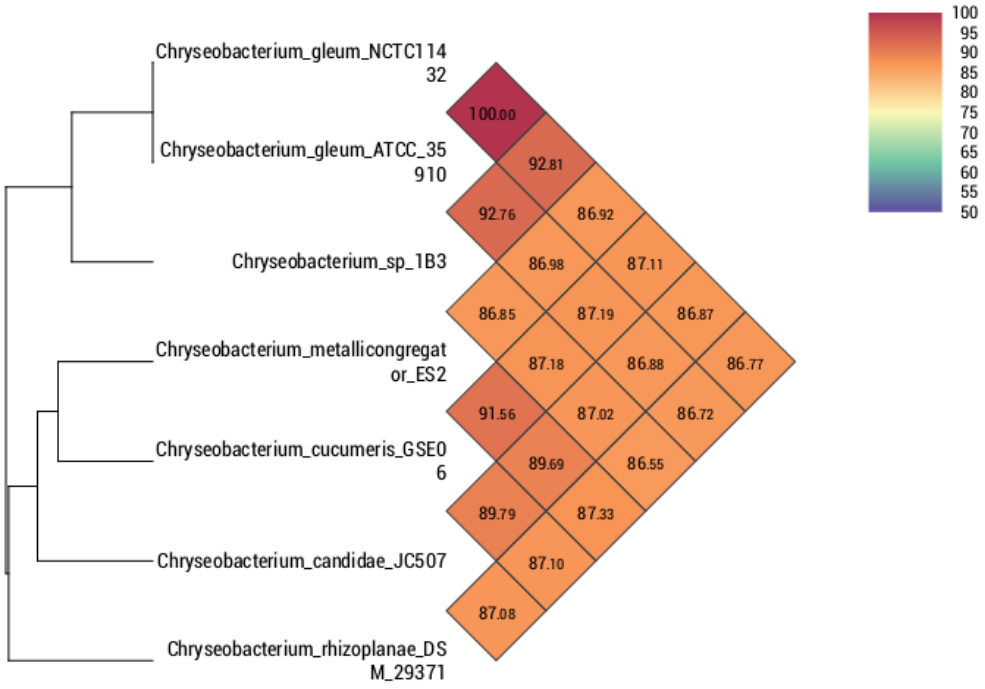

Figure 4.4 Average Nucleotide Identities of *Chryseobacterium sp.* 1B3.

Pseudomonas sp. 2A5 was isolated using *Janthinobacterium sp.* 2022 as bait. It has a genome size of 6,042,350 base pairs. The GC content is 59.61% (Table 5.3). There were 5,975 protein coding sequences and 59 RNA genes predicted. *Pseudomonas sp.* 2A5 had the highest ANI (92.45%) to *Pseudomonas grandcourensis*, which was originally isolated from the rhizosphere of wheat in Switzerland (Poli, Keel, and Garrido-Sanz 2024). *Pseudomonas sp.* 2A5 shared 2,547 (30.05%) sequences with *Pseudomonas grandcourensis*, while 5,928 (69.95%) were not shared (Table 4.1).

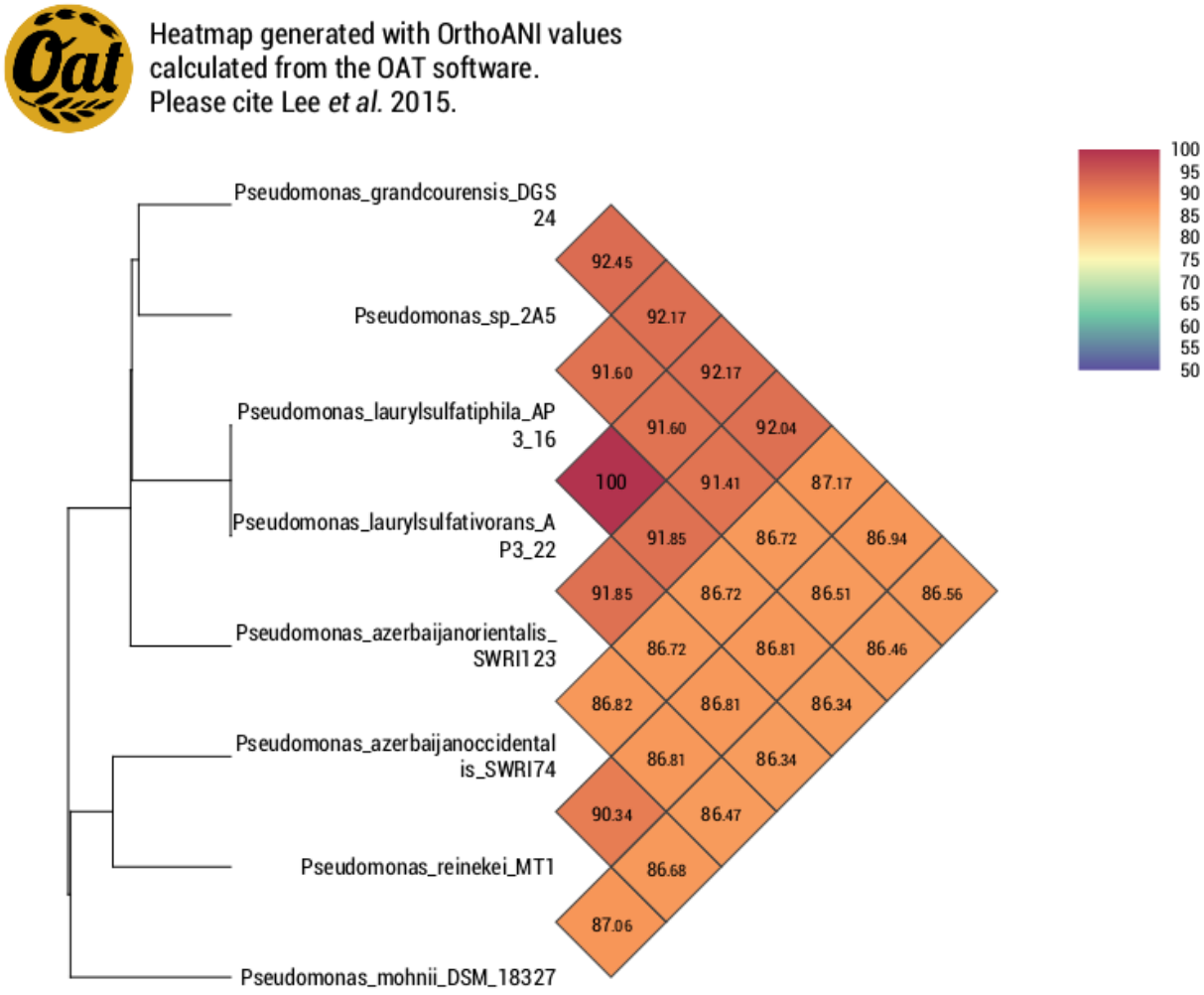

Figure 4.5 Average Nucleotide Identities of *Pseudomonas sp.* 2A5.

Variovorax sp. 2A5 was isolated using *Janthinobacterium sp.* 2022 as bait. It has a genome size of 5,965,519 base pairs in one chromosome and the GC content is 67.7 (Table 4.3). There were 6,637 protein coding sequences and 67 RNA genes. *Variovorax sp.* 2A5 had the highest ANI score to *Variovorax guangxiensis* at 93.59%. *Variovorax guangxiensis* was originally isolated from the rhizosphere of banana plants in China (Gao et al. 2015). *Variovorax sp.* 2A5 shares 3,695 (39.06%) genes with *Variovorax guangxiensis*, while 5,765 (60.94%) were not shared (Table 4.1).

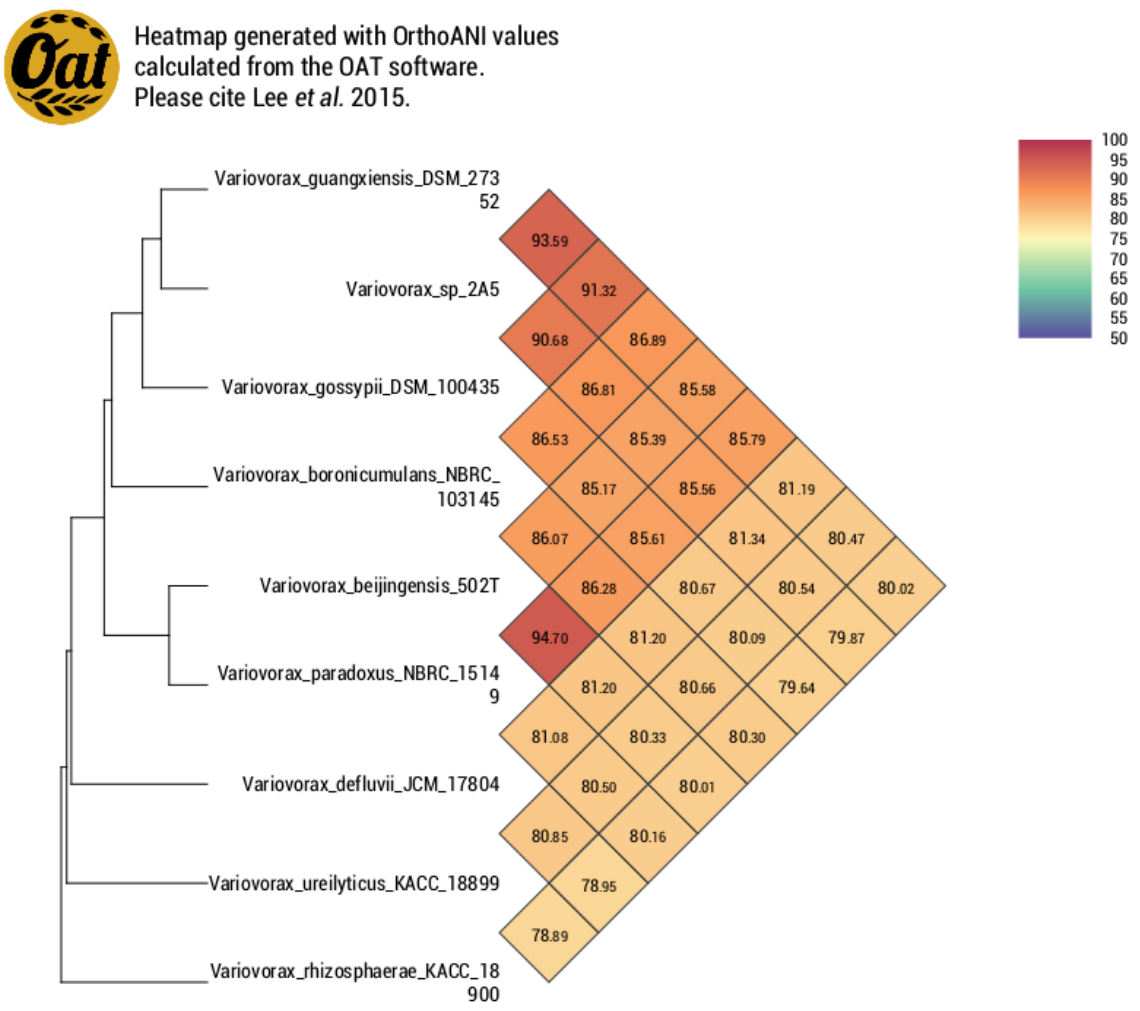

Figure 4.6 Average Nucleotide Identities of *Variovorax sp.* 2A5.

Sorghum field soil

Pseudomonas sp. 3-2 has a genome size of 6,263,213 base pairs in one chromosome, and the GC content is 60.8% (Table 4.3). There were 5,877 protein coding sequences and 62 RNA genes predicted. *Pseudomonas sp.* 3-2 has the highest ANI (94.85) to *Pseudomonas aphyarum*, which was originally isolated from a fish hatchery in Idaho (Testerman et al. 2023). *Pseudomonas sp.* 3-2 shares 4,457 (62.93%) genes with *Pseudomonas aphyarum*, while 2,626 (37.07%) were not shared (Table 4.1).

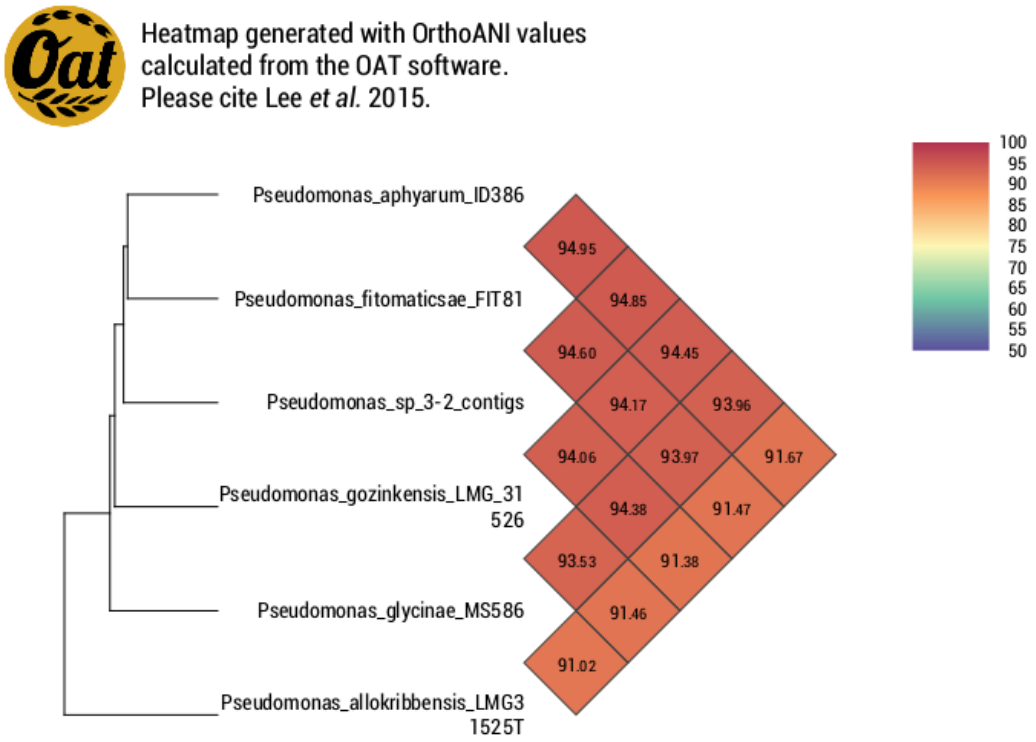

Figure 4.7 Average Nucleotide Identities of *Pseudomonas sp.* 3-2

Mangrove associated bacteria

Two isolates from mangrove were suggested as new species by TYGS and confirmed by Average Nucleotide Identity scores (Table 4.1).

Mitsuaria sp. S21 has a genome size of 6,067,743 base pairs and is composed of 1 contig (Table 4.1). The GC content is 69.8% (Table 4.1). There were 5,484 protein coding sequences and 76 RNA genes predicted. The most closely related species is *Mitsuaria chitinivorans* (Figure 4.8) originally isolated from a tube well in f Hyderabad, India (Fan et al. 2018). There are 2,819 (37.21%) genes shared between *Mitsuaria sp.* S21 and *Mitsuaria chitinivorans*, and 4,756 genes (62.79%) unshared (Table 4.1).

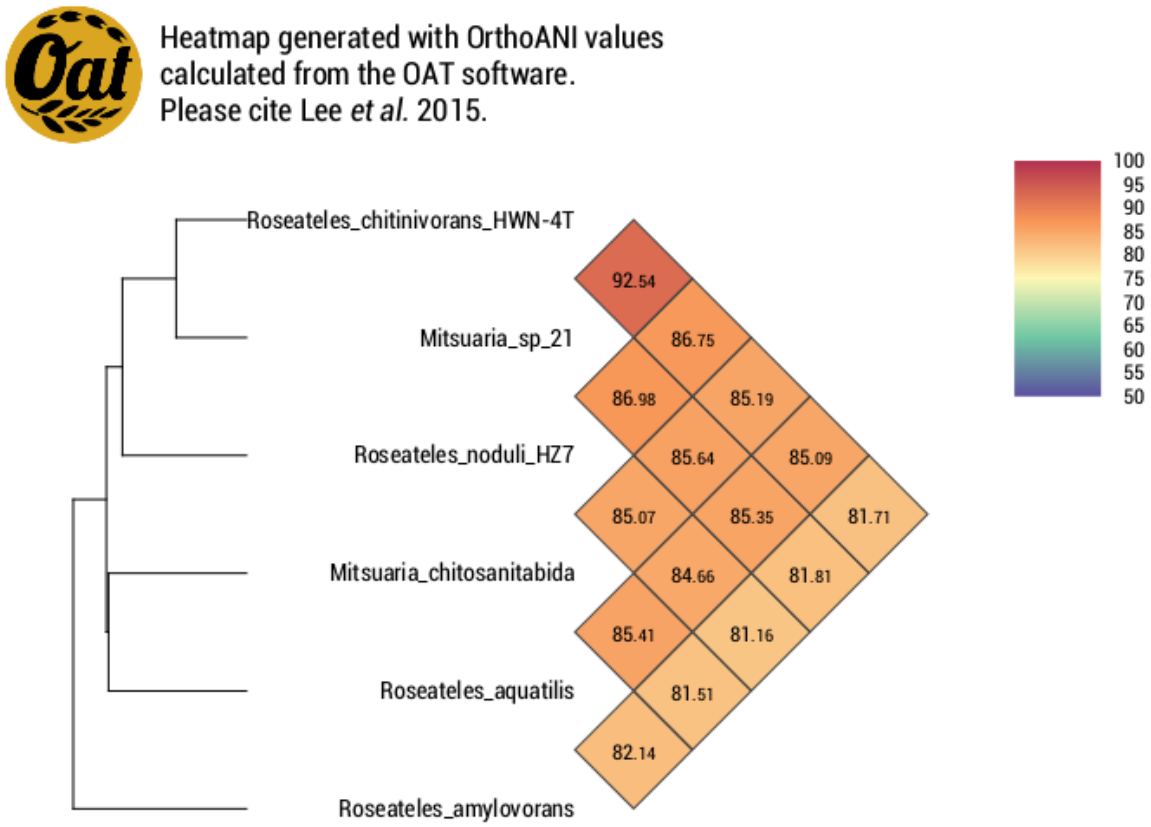

Figure 4.8 Average Nucleotide Identities of *Mitsuaria sp.* S21

Pseudomonas sp. S32 has a genome size of 5,772,064 base pairs and is composed of 2 contigs and the GC content is 61.6% (Table 4.1). There were 5,595 protein coding sequences and 79 RNA genes identified. *Pseudomonas sp.* S32 had the highest ANI score (89.85%) to *Pseudomonas putida* (Figure 4.9) a well-known and ubiquitous soil bacteria that is often considered a "model microbe" for soil bacterial studies (Belda et al. 2016). *Pseudomonas sp.* S32 shared 3,007 (33.66%) *Pseudomonas putida*, and 5926 (66.34%) were not shared (Table 4.1).

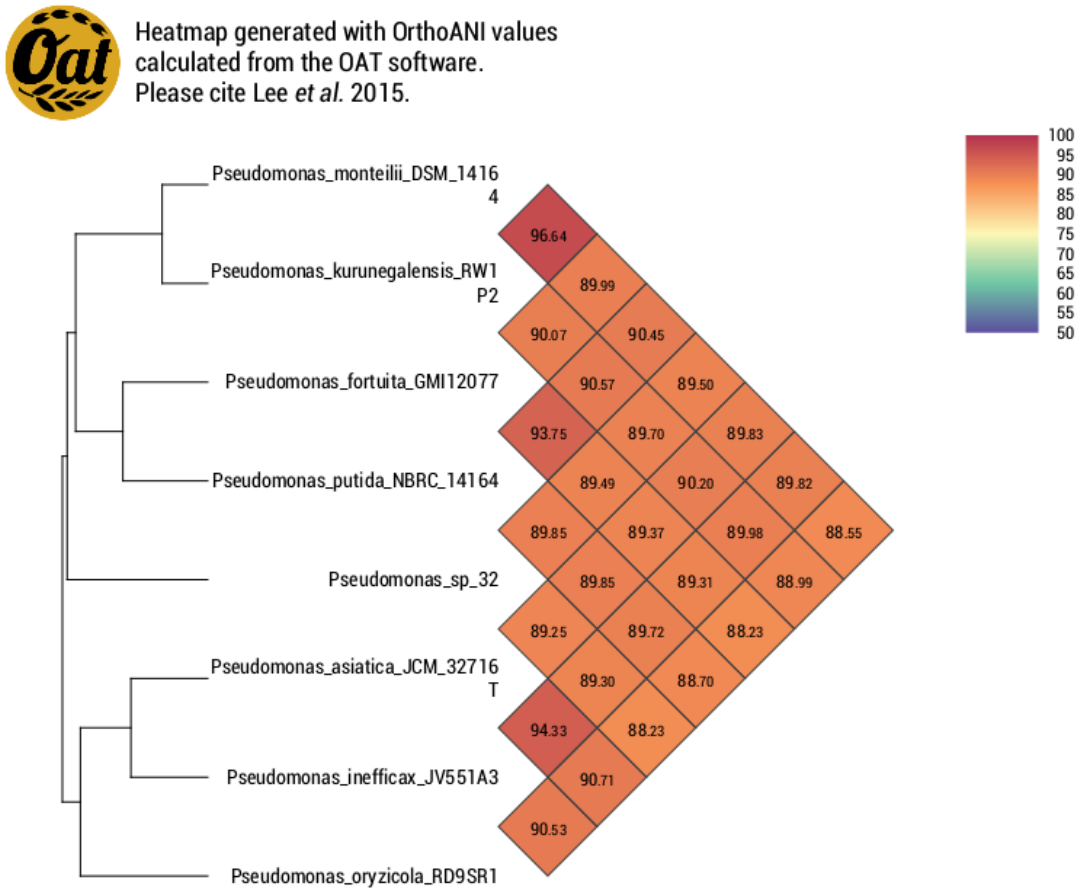

Figure 4.9 Average Nucleotide Identities of *Pseudomonas sp.* S32

Sorghum rhizoplane

Pseudomonas sp. SR04 has a genome size of 7,058,539 base pairs and is composed of 7 contigs, and the GC content is 61.6% (Table 4.1). There were 6,095 protein coding sequences and 77 RNA genes identified. *Pseudomonas sp.* SR04 had the highest ANI score (93.40%) to *Pseudomonas beijingensis*, which was originally isolated from the rhizosphere of rice (Figure 4.10). *Pseudomonas sp.* SR04 shared 3,798 (46.92%) genes with *Pseudomonas beijingensis*, while 4,296 (53.08%) were not shared (Table 4.1).

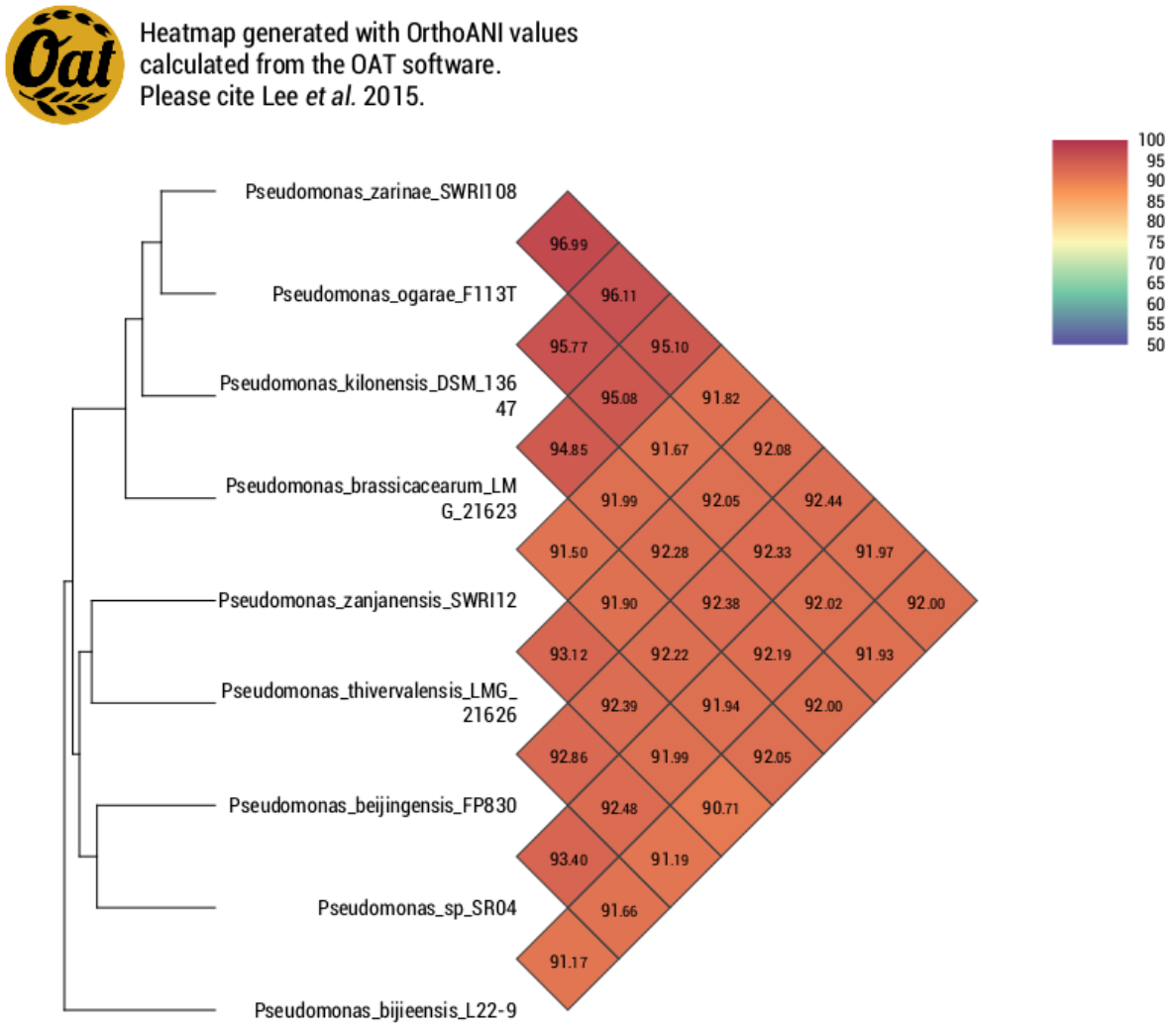

Figure 4.10 Average Nucleotide Identities of *Pseudomonas sp.* SR04.

Discussion

Soil and plant roots are known to harbor a diverse array of microbial life, including bacteria that play critical roles in nutrient cycling, plant health, and soil structure. In the case of soil, it is the most diverse microbial habitat on Earth, harboring at least one-quarter of its total biodiversity (Sokol et al. 2022). Microbial diversity encompasses the complexity and variability across various biological levels, including genetic differences within species, and the number (richness) and relative abundance (evenness) of species, and functional groups within communities (Torsvik and

Øvreås 2002). Both, the soil and plant roots serve as a reservoir for discovering new bacterial species, many of which remain uncharacterized (Ramirez et al. 2014). The unique conditions within the soil and root zones, such as varying moisture levels, nutrient availability, and plant exudates, create niches where different bacteria can thrive (Trivedi et al. 2020; Sokol et al. 2022). This diversity offers an opportunity for researchers to identify novel bacterial species that may have unique capabilities for improving soil health, promoting plant growth, or even contributing to biotechnological applications.

Recent advancements in genomics and methodological approaches have significantly enhanced our ability to discover and characterize new bacterial species, as well as to explore soil microbial diversity and its influence on soil processes (Fierer 2017). Genomic sequencing enables researchers to analyze bacterial DNA directly from environmental samples, increasing our knowledge of bacterial diversity without the need for cultivation. This provides critical insights into their genetic potential, evolutionary relationships, and metabolic pathways. This approach circumvents the traditional requirement for laboratory cultivation of bacteria, which has historically limited the discovery of new species due to challenges in replicating their natural growth conditions. This has also facilitated species discovery from cultivated samples, helping to classify and reclassify species, break genera into sister genera, and describe cryptic species (Konstantinidis, Ramette, and Tiedje 2006; Lee et al. 2016; Konstantinidis 2023). As a result, genomics has opened a new frontier for microbial research, revealing a previously hidden world of microbial diversity within soil and root ecosystems.

Despite the advantages of the use of Average Nucleotide Identity for the delimitation of species, some findings have challenged earlier conclusions based on DNA-DNA hybridization (DDH) (Konstantinidis 2023). During the last two decades, genomes with intermediate levels of

genomic similarity (such as 90%–95% Average Nucleotide Identity (ANI) corresponding to 30%–70% DDH) and overlapping phenotypes compared to known species have been frequently identified (Konstantinidis 2023). A notable example is the *Escherichia coli* group , where numerous genomes with characteristic *E. coli* phenotypes (like lactose fermentation) became available, showing ANI values in the 90%–92% range compared to *E. coli* reference genomes (Luo et al. 2011). These observations have led some scientists to question the existence of distinct bacterial species and suggest that species boundaries are, at best, "fuzzy" (Konstantinidis 2023).

Jain et al. (2018) examined 90,000 bacterial genomes from the NCBI database, and found that approximately 97% of described (named) species include genomes demonstrating >95% ANI among themselves, while showing <86% ANI to representatives of other species. This observation aligns with the 70% DDH definition of species and highlights a striking similarity between the results of isolated genome comparisons and earlier metagenomic findings, contrasting sharply with earlier conclusions derived from genome sequencing of bacterial isolates (Konstantinidis 2023). The differences observed may stem from the fact that previous studies often compared organisms adapted to distinct ecological niches, which obscured the delineation of discrete species (Konstantinidis, 2023). The concept of species in bacteria remains a topic of ongoing debate, but the 95% ANI cutoff has proven to be useful and is broadly used in the study of bacterial diversity.

It has been previously shown that even soil in a highly impacted urban environment contains undescribed bacterial species (Ramirez et al. 2014). *Janthinobacterium sp.* 2021 came from soil near a parking lot on the campus of the University of Georgia. Ramirez et al. (2014), found that the soils of Central Park in New York City harbored nearly as many distinct microbial phylotypes and community types as those found in biomes across the globe, including arctic, tropical, and desert soils. This study highlighted that the amount and patterning of novel and

uncharacterized diversity in a single urban location could match that observed across natural ecosystems spanning multiple biomes and continents. This shows the importance of exploring the microbial diversity in urban soils, and their potential to host new undescribed species.

The isolation of bacteria from desiccated soil was conducted as part of an undergraduate research project aimed at exploring desiccated soil as a source for bacterial isolation. This approach is particularly relevant given that reductions in soil moisture can significantly impact microbial communities, which rely on water within soil pores for their life cycles and essential functions (Vos et al. 2013). In conditions of low soil water availability, desiccation-tolerant groups tend to outcompete those less adapted, causing a shift in the lifestyle of prokaryotic taxa from copiotrophic to oligotrophic (Jaeger et al. 2024). *Mucilaginibacter sp.* DS21 is a prime example of bacteria adapted to desiccated soil. Members of the genus *Mucilaginibacter* are recognized for their ability to produce exopolysaccharides (EPS) (Kumar, Mukhia, and Kumar 2022), a characteristic closely associated with drought resistance in bacteria (Roberson and Firestone 1992). EPS production not only enhances the bacterium's ability to retain moisture but also plays a crucial role in forming more complex biofilms, which can assist microbial community survival under harsh environmental conditions (Bogino et al. 2013; Roberson and Firestone 1992). By investigating the microbial diversity present in desiccated soils, this project contributes to our understanding of how bacterial communities adapt to extreme environments and the ecological functions they perform within these contexts.

It has been reported that most soil bacteria are recalcitrant to grow as pure cultures on a petri dish, with many authors estimating that only about 1% of soil bacteria can be successfully cultured, leaving an astonishing 99% uncultured (Chaudhary, Khulan, and Kim 2019; Pham and Kim 2012; Kirk et al. 2004). This large proportion of "unculturable" bacteria represents a vast,

untapped reservoir of species with potentially unique biological and chemical properties (Chaudhary, Khulan, and Kim 2019). While these bacteria are metabolically active in their natural environments, they cannot grow in laboratory media. The term "unculturable" is somewhat misleading, as it does not imply that these organisms can never be cultured. Instead, it reflects our limited understanding of their habitats, abiotic and biotic interactions, and ecological roles in soil. One of the main reasons for this is the inability to replicate their natural growth conditions. Additionally, insufficient knowledge about crucial factors such as generation time, optimal growth temperature, and nutrient requirements contributes to the unculturability of certain bacteria (Chaudhary, Khulan, and Kim 2019; Pham and Kim 2012; Stewart 2012). New techniques for bacterial isolation such as the use of microbial partners (MiPners), can further enhance our capacity to isolate and discover new bacterial species from both soil and roots. Although MiPner technology was designed for isolating pairwise microbial partnerships based on their physical associations through microbe-microbe binding, the first MiPner experiments uncovered a high number of potential new species, as it can be shown in my results.

Red mangroves, which are the most ocean-exposed type of mangrove, play a crucial role in coastal protection across tropical regions (DeYoe et al. 2020). They root in soils that are usually submerged in water with salt concentrations ranging from less than 0.1% NaCl—during periods of high river runoff and low tides—to over 3% NaCl when tides flow into estuaries or coastal ponds that begin to evaporate after the tides recede. Although little is known about the microbes that interact with mangroves, initial studies suggest that some of these microorganisms may aid mangroves (and potentially other plants) in coping with high salinity levels (Soldan et al. 2019). Notably, both *Mitsuaria sp.* 21 and *Pseudomonas sp.* 32 have the potential to encode genes that could help plants cope with salt stress. These genes may be involved in various mechanisms, such

as enhancing osmotic adjustment, which allows plants to maintain cell turgor pressure despite high salinity levels. Understanding the genetic and functional traits of these and other microbial species from mangroves, could provide valuable insights into their role in fostering plant adaptation to saline conditions, ultimately aiding in the conservation and management of mangrove ecosystems.

Many of these microbes were isolated as part of the course GENE4240L, a hands-on laboratory experience that emphasizes active participation in real scientific research. This course embodies the principles of Course-based Undergraduate Research Experiences (CUREs). CUREs represent an innovative type of classroom-based course that provides students with practical experience in conducting original research while allowing faculty to produce new knowledge within their field (Szteinberg and Weaver 2013; Bangera and Brownell 2014).

In the context of GENE4240L, students not only engage with the field of microbiology but also contribute to significant scientific advancements. By isolating, identifying, and characterizing new microbial species, students acquire essential practical skills in microbiology and genomics. In addition to this, the experience of CUREs has been shown to fosters enhanced self-confidence in scientific thinking and helps develop vital scientific process skills (Szteinberg and Weaver 2013; Brownell et al. 2015). Additionally, CUREs promote inclusivity in science, particularly for underrepresented populations, and have been shown to improve persistence in science and medicine (Bangera and Brownell 2014; Hanauer et al. 2012).

Moreover, the integration of CUREs into the undergraduate curriculum illustrates how academic research can transcend traditional classroom boundaries. The insights gained from these research activities can lead to the identification of novel bacterial species, which may hold significant ecological or biotechnological importance. Such discoveries can have profound

implications, potentially contributing to advancements in fields like environmental sustainability, healthcare, and biotechnology.

CHAPTER 5

DISCUSSION

This project explores the diverse roles of genomics in studying microbial communities in soil and roots, aiming to deepen our understanding of the composition, function, and dynamics of these microbial communities. Soil is the most biologically diverse microbial habitat on the planet, containing roughly a quarter of global biodiversity (Quince, Curtis, and Sloan 2008; Sokol et al. 2022; Vos et al. 2013). Soil-borne microorganisms are essential to numerous ecosystem functions, including nutrient cycling, plant health, water purification, carbon storage, and the maintenance of soil structure. As human reliance on these services increases (Young and Crawford 2004), understanding the biology of soil microbiomes becomes increasingly critical.

This project required metagenomic sequencing of soil and bacterial collections on plates, plus long read sequencing of individual bacterial isolates. While metagenomic sequencing allows for the analysis of environmental samples to provide a taxonomic profile (Daniel 2005), the sequencing of individual isolates with long read technologies like Oxford Nanopore allows one to obtain chromosome level assemblies, allows the identification of plasmids, and can lead to precise characterization of individual genomes (Sereika et al. 2022). Looking at individual genomes allows a more detailed understanding of the biology of the bacteria investigated. Due to the high percentage of genes that could not be grouped into subsystems, as shown in the second chapter, studies of individual bacterial genomes can shed light into the unknown genetic diversity of soil and plant-associated genomes.

Despite the limitations of culture-based methods, which typically enable the isolation of a small percentage of soil and plant associated bacteria (Chaudhary, Khulan, and Kim 2019; Pham and Kim 2012; Kirk et al. 2004), these traditional approaches remain valuable for the discovery of new bacterial species. Though culture-independent techniques have advanced significantly, they still offer only limited insights into the physiology of prokaryotes (Rosenberg et al. 2013). The role of not-yet-cultured prokaryotes in the environment cannot be fully understood until these microorganisms are available for detailed physiological and molecular studies (Rosenberg et al. 2013).

Traditionally, the vast majority of microbial analyses have been performed one species at a time. Recently developed technologies, grouped under the term microbiomics, have allowed considerations of very large environmental communities, mostly at a descriptive level. The combination of traditional culture-based methods with the newly developed Microbial Partner (MiPner) technique provides a first step toward bridging this gap. Studying two species together is much more complex than one at a time. Hence, using a criterion (evolved specific binding) that suggests these two bacterial species actually interact in nature is thus appropriate before one takes the step in two-species analysis.

Future research on the MiPners technique could involve using one of the isolated MiPners as bait. Since these microbes have shown the ability to bind to *Serratia marcescens*, it should be expected that *S. marcescens* will bind them if it is present in the soil. This approach could be expanded by combining the MiPner with *S. marcescens* as a dual bait system. Further studies could also include sequencing the microorganisms that bind directly to the sticks in MiPners, without culturing and the comparing that community to what grows on petri dishes.

Beyond *Serratia marcescens*, this approach can be applied to other bacterial species isolated and characterized in this thesis, such as *Janthinobacterium* (known for its purple colonies), *Pantoea*, *Flavobacterium*, *Sphingomonas*, and *Chryseobacterium* (which produce yellow colonies). Using these bacteria could extend the technique's application to root environments, contributing to a deeper understanding of the microbiomes of the soil and the rhizoplane.

The study of rhizoplane bacteria has highlighted how much remains unknown about the communities in this root compartment. Among the ten bacteria sequenced, six are new species, and only a small proportion of their genes could be categorized into subsystems using RAST. Notably, four out of six subsystems showed significant gene enrichment in the rhizoplane isolates. Compared to the rhizosphere and endosphere, the rhizoplane has received relatively little attention. Analyzing the genomes of these ten isolates is a important step towards a better understanding of this root compartment. These isolates could be used for controlled inoculation experiments, biofilm formation assays, and the knockout of genes potentially involved in root colonization. Future research should focus on increasing the number of isolates from the rhizoplane, encompassing both maize and other plant species, while also expanding the diversity of taxonomic groups represented.

In this dissertation, the second chapter characterizes six new bacterial species isolated from the maize rhizoplane. The third chapter focuses on the isolation and description of two potential new species, while the fourth chapter presents the characterization of eleven new species. Defining bacterial species is key for identification, diagnostics, and biodiversity assessments, yet it has long been a complex challenge (Konstantinidis, Ramette, and Tiedje 2006). Genomic approaches offer new insights into diversity within species, providing opportunities to develop a more robust classification system (Konstantinidis, Ramette, and Tiedje 2006). As previously mentioned, the

genome remains the ultimate source of taxonomic information (Lee et al. 2016). The use of a 95% Average Nucleotide Identity (ANI) cutoff for defining bacterial species provides an operational species definition that facilitates the automated assignment of genomes to species. This method scales effectively to large datasets, enabling the organization of all current and future genomes into species clusters (Parks et al. 2020).

Beyond aiding in the discovery of new species, the MiPner technique offers a valuable tool for developing synthetic microbial communities. Many efforts have been made to construct synthetic communities (Liu, Qin, and Bai 2019; Niu et al. 2017; Großkopf and Soyer 2014; Johns et al. 2016; Martins et al. 2023), but these efforts face challenges in selecting microorganisms that accurately represent the taxonomic composition of the target microbiome and in replicating natural microbe-microbe interactions (Jing et al. 2024; Großkopf and Soyer 2014). The isolation and characterization of bacteria from soil and roots are crucial for selecting microbes that reflect the composition of the microbiome under study. When it comes to replicating natural interactions, MiPners can serve as a starting point, as they inherently require pairwise interactions. Thus, the microbes identified and sequenced in this work could be used to build synthetic communities derived from root- and soil-associated microbes.

Many microbes face challenges in synthetic communities due to the complex and delicate interdependencies typical of natural microbial ecosystems (Großkopf and Soyer 2014). These species often rely on specific metabolic interactions, nutrient exchanges, and environmental cues that are difficult to replicate in simplified synthetic environments. This can lead to imbalances, increased competition, and an inability for some microbes to survive without their natural partners (Großkopf and Soyer 2014; Jing et al. 2024; Johns et al. 2016). The MiPner approach, which

emphasizes the interaction between two microbes binding to each other, can serve as a foundational step for future experiments aimed at developing synthetic communities.

In conclusion, this project demonstrates the multifaceted role of genomics in studying microbiomes of soil and plant. From the use of metagenomics to look at soil and plates, to the study of bacteria-bacteria interactions with the microbial partners (MiPner technique), the study of rhizoplane associated bacteria and the advancement of new bacterial species discovery through genome-enabled taxonomy, the study provides valuable insights into the complex relationships between plants and their microbial partners. These findings not only deepen our understanding of bacterial biology but also pave the way for future applications in agriculture, environmental science, and biotechnology.

REFERENCES

Adams, Mark D., Jenny M. Kelley, Jeannine D. Gocayne, Mark Dubnick, Mihael H. Polymeropoulos, Hong Xiao, Carl R. Merril, et al. 1991. "Complementary DNA Sequencing: Expressed Sequence Tags and Human Genome Project." *Science* 252 (5013): 1651–56.

Alikhan, Nabil-Fareed, Nicola K Petty, Nouri L Ben Zakour, and Scott A Beatson. 2011. "BLAST Ring Image Generator (BRIG): Simple Prokaryote Genome Comparisons." *BMC Genomics* 12 (1): 402. https://doi.org/10.1186/1471-2164-12-402.

Allard-Massicotte, Rosalie, Laurence Tessier, Frédéric Lécuyer, Venkatachalam Lakshmanan, Jean-François Lucier, Daniel Garneau, Larissa Caudwell, Hera Vlamakis, Harsh P. Bais, and Pascale B. Beauregard. 2016. "*Bacillus Subtilis* Early Colonization of *Arabidopsis Thaliana* Roots Involves Multiple Chemotaxis Receptors." Edited by Paul D. Straight and Edward G. Ruby. *mBio* 7 (6): e01664-16. https://doi.org/10.1128/mBio.01664-16.

Alvarez Crespo, María Cecilia, and Claudio Valverde. 2009. "A Single Mutation in the oprF mRNA Leader Confers Strict Translational Control by the Gac/Rsm System in *Pseudomonas Fluorescens* CHA0." *Current Microbiology* 58 (2): 182–88. https://doi.org/10.1007/s00284-008-9306-6.

Andrés-Barrao, Cristina, Hanin Alzubaidy, Rewaa Jalal, Kiruthiga G. Mariappan, Axel De Zélicourt, Ameerah Bokhari, Olga Artyukh, et al. 2021. "Coordinated Bacterial and Plant Sulfur Metabolism in *Enterobacter* Sp. SA187–Induced Plant Salt Stress Tolerance."

Proceedings of the National Academy of Sciences 118 (46): e2107417118. https://doi.org/10.1073/pnas.2107417118.

Aulakh, M. S., R. Wassmann, C. Bueno, J. Kreuzwieser, and H. Rennenberg. 2001. "Characterization of Root Exudates at Different Growth Stages of Ten Rice (*Oryza Sativa* L.) Cultivars." *Plant Biology* 3 (2): 139–48. https://doi.org/10.1055/s-2001-12905.

Aziz, Ramy K, Daniela Bartels, Aaron A Best, Matthew DeJongh, Terrence Disz, Robert A Edwards, Kevin Formsma, et al. 2008. "The RAST Server: Rapid Annotations Using Subsystems Technology." *BMC Genomics* 9 (1): 75. https://doi.org/10.1186/1471-2164-9-75.

Bangera, Gita, and Sara E. Brownell. 2014. "Course-Based Undergraduate Research Experiences Can Make Scientific Research More Inclusive." Edited by Graham Hatfull. *CBE—Life Sciences Education* 13 (4): 602–6. https://doi.org/10.1187/cbe.14-06-0099.

Banks, M K, and J D Bryers. 1991. "Bacterial Species Dominance within a Binary Culture Biofilm." *Applied and Environmental Microbiology* 57 (7): 1974–79. https://doi.org/10.1128/aem.57.7.1974-1979.1991.

Bapteste, Eric, Maureen A O'Malley, Robert G Beiko, Marc Ereshefsky, J Peter Gogarten, Laura Franklin-Hall, François-Joseph Lapointe, et al. 2009. "Prokaryotic Evolution and the Tree of Life Are Two Different Things." *Biology Direct* 4 (1): 34. https://doi.org/10.1186/1745-6150-4-34.

Bardgett, Richard D., Liesje Mommer, and Franciska T. De Vries. 2014. "Going Underground: Root Traits as Drivers of Ecosystem Processes." *Trends in Ecology & Evolution* 29 (12): 692–99. https://doi.org/10.1016/j.tree.2014.10.006.

Bashan, Yoav, Luz E. de-Bashan, S. R. Prabhu, and Juan-Pablo Hernandez. 2014. "Advances in Plant Growth-Promoting Bacterial Inoculant Technology: Formulations and Practical Perspectives (1998–2013)." *Plant and Soil* 378 (1–2): 1–33. https://doi.org/10.1007/s11104-013-1956-x.

Beauregard, Pascale B., Yunrong Chai, Hera Vlamakis, Richard Losick, and Roberto Kolter. 2013. "*Bacillus Subtilis* Biofilm Induction by Plant Polysaccharides." *Proceedings of the National Academy of Sciences* 110 (17). https://doi.org/10.1073/pnas.1218984110.

Beaz-Hidalgo, Roxana, Mohammad J. Hossain, Mark R. Liles, and Maria-Jose Figueras. 2015. "Strategies to Avoid Wrongly Labelled Genomes Using as Example the Detected Wrong Taxonomic Affiliation for Aeromonas Genomes in the GenBank Database." Edited by Turgay Unver. *PLOS ONE* 10 (1): e0115813. https://doi.org/10.1371/journal.pone.0115813.

Belda, Eugeni, Ruben G. A. Van Heck, Maria José Lopez-Sanchez, Stéphane Cruveiller, Valérie Barbe, Claire Fraser, Hans-Peter Klenk, et al. 2016. "The Revisited Genome of *Pseudomonas Putida* KT2440 Enlightens Its Value as a Robust Metabolic *Chassis*." *Environmental Microbiology* 18 (10): 3403–24. https://doi.org/10.1111/1462-2920.13230.

Bell, Jennifer K., Steven D. Mamet, Bobbi Helgason, and Steven D. Siciliano. 2022. "*Brassica Napus* Bacterial Assembly Processes Vary with Plant Compartment and Growth Stage but Not between Lines." Edited by Isaac Cann. *Applied and Environmental Microbiology* 88 (10): e00273-22. https://doi.org/10.1128/aem.00273-22.

Berne, Cécile, Adrien Ducret, Gail G. Hardy, and Yves V. Brun. 2015. "Adhesins Involved in Attachment to Abiotic Surfaces by Gram-Negative Bacteria." Edited by Mahmoud

Ghannoum, Matthew Parsek, Marvin Whiteley, and Pranab Mukherjee. *Microbiology Spectrum* 3 (4): 3.4.15. https://doi.org/10.1128/microbiolspec.MB-0018-2015.

Bertoldo, Giovanni, Maria Cristina Della Lucia, Andrea Squartini, Giuseppe Concheri, Chiara Broccanello, Alessandro Romano, Samathmika Ravi, Massimo Cagnin, Andrea Baglieri, and Piergiorgio Stevanato. 2021. "Endophytic Microbiome Responses to Sulfur Availability in *Beta Vulgaris* (L.)." *International Journal of Molecular Sciences* 22 (13): 7184. https://doi.org/10.3390/ijms22137184.

Blair, Kris M., Linda Turner, Jared T. Winkelman, Howard C. Berg, and Daniel B. Kearns. 2008. "A Molecular Clutch Disables Flagella in the *Bacillus Subtilis* Biofilm." *Science* 320 (5883): 1636–38. https://doi.org/10.1126/science.1157877.

Bobay, Louis-Marie. 2020. "The Prokaryotic Species Concept and Challenges." In *The Pangenome*, edited by Hervé Tettelin and Duccio Medini, 21–49. Cham: Springer International Publishing. https://doi.org/10.1007/978-3-030-38281-0_2.

Bogino, Pablo, María Oliva, Fernando Sorroche, and Walter Giordano. 2013. "The Role of Bacterial Biofilms and Surface Components in Plant-Bacterial Associations." *International Journal of Molecular Sciences* 14 (8): 15838–59. https://doi.org/10.3390/ijms140815838.

Bolotin, Evgeni, and Ruth Hershberg. 2016. "Bacterial Intra-Species Gene Loss Occurs in a Largely Clocklike Manner Mostly within a Pool of Less Conserved and Constrained Genes." *Scientific Reports* 6 (1): 35168. https://doi.org/10.1038/srep35168.

Bonkowski, Michael, Mika Tarkka, Bahar S. Razavi, Hannes Schmidt, Evgenia Blagodatskaya, Robert Koller, Peng Yu, Claudia Knief, Frank Hochholdinger, and Doris Vetterlein. 2021. "Spatiotemporal Dynamics of Maize (*Zea Mays* L.) Root Growth and Its

Potential Consequences for the Assembly of the Rhizosphere Microbiota." *Frontiers in Microbiology* 12 (March):619499. https://doi.org/10.3389/fmicb.2021.619499.

Bourceret, Amelia, Rui Guan, Kristof Dorau, Tim Mansfeldt, Amin Omidbakhshfard, David B. Medeiros, Alisdair R. Fernie, et al. 2022. "Maize Field Study Reveals Covaried Microbiota and Metabolic Changes in Roots over Plant Growth." Edited by Ann M. Hirsch and Margaret J. McFall-Ngai. *mBio* 13 (2): e02584-21. https://doi.org/10.1128/mbio.02584-21.

Breugelmans, Philip, Kim Bundvig Barken, Tim Tolker-Nielsen, Johan Hofkens, Winnie Dejonghe, and Dirk Springael. 2008. "Architecture and Spatial Organization in a Triple-Species Bacterial Biofilm Synergistically Degrading the Phenylurea Herbicide Linuron." *FEMS Microbiology Ecology* 64 (2): 271–82.

Brownell, Sara E., Daria S. Hekmat-Scafe, Veena Singla, Patricia Chandler Seawell, Jamie F. Conklin Imam, Sarah L. Eddy, Tim Stearns, and Martha S. Cyert. 2015. "A High-Enrollment Course-Based Undergraduate Research Experience Improves Student Conceptions of Scientific Thinking and Ability to Interpret Data." Edited by James Hewlett. *CBE—Life Sciences Education* 14 (2): ar21. https://doi.org/10.1187/cbe.14-05-0092.

Bulgarelli, Davide, Ruben Garrido-Oter, Philipp C. Münch, Aaron Weiman, Johannes Dröge, Yao Pan, Alice C. McHardy, and Paul Schulze-Lefert. 2015. "Structure and Function of the Bacterial Root Microbiota in Wild and Domesticated Barley." *Cell Host & Microbe* 17 (3): 392–403. https://doi.org/10.1016/j.chom.2015.01.011.

Bulgarelli, Davide, Klaus Schlaeppi, Stijn Spaepen, Emiel Ver Loren Van Themaat, and Paul Schulze-Lefert. 2013. "Structure and Functions of the Bacterial Microbiota of Plants."

Annual Review of Plant Biology 64 (1): 807–38. https://doi.org/10.1146/annurev-arplant-050312-120106.

Burdman, Saul, Gabriella Dulguerova, Yaacov Okon, and Edouard Jurkevitch. 2001. "Purification of the Major Outer Membrane Protein of *Azospirillum Brasilense* , Its Affinity to Plant Roots, and Its Involvement in Cell Aggregation." *Molecular Plant-Microbe Interactions*® 14 (4): 555–61. https://doi.org/10.1094/MPMI.2001.14.4.555.

Burmølle, Mette, Kaare Johnsen, Waleed Abu Al-Soud, Lars Hestbjerg Hansen, and Søren Johannes Sørensen. 2009. "The Presence of Embedded Bacterial Pure Cultures in Agar Plates Stimulate the Culturability of Soil Bacteria." *Journal of Microbiological Methods* 79 (2): 166–73. https://doi.org/10.1016/j.mimet.2009.08.006.

Bushnell, B. 2014. "BBMap: A Fast, Accurate, Splice-Aware Aligner."

Carini, Paul, Patrick J. Marsden, Jonathan W. Leff, Emily E. Morgan, Michael S. Strickland, and Noah Fierer. 2016. "Relic DNA Is Abundant in Soil and Obscures Estimates of Soil Microbial Diversity." *Nature Microbiology* 2 (3): 16242. https://doi.org/10.1038/nmicrobiol.2016.242.

Caro-Quintero, Alejandro, and Konstantinos T. Konstantinidis. 2012. "Bacterial Species May Exist, Metagenomics Reveal." *Environmental Microbiology* 14 (2): 347–55.

Carr, Alex, Christian Diener, Nitin S Baliga, and Sean M Gibbons. 2019. "Use and Abuse of Correlation Analyses in Microbial Ecology." *The ISME Journal* 13 (11): 2647–55. https://doi.org/10.1038/s41396-019-0459-z.

Chaluvadi, Srinivasa, and Jeffrey L. Bennetzen. 2018. "Species-Associated Differences in the Below-Ground Microbiomes of Wild and Domesticated *Setaria*." *Frontiers in Plant Science* 9 (August):1183. https://doi.org/10.3389/fpls.2018.01183.

Chaudhary, Dhiraj Kumar, Altankhuu Khulan, and Jaisoo Kim. 2019. “Development of a Novel Cultivation Technique for Uncultured Soil Bacteria.” *Scientific Reports* 9 (1): 6666. https://doi.org/10.1038/s41598-019-43182-x.

Chaudhry, Vasvi, Paul Runge, Priyamedha Sengupta, Gunther Doehlemann, Jane E Parker, and Eric Kemen. 2021. “Shaping the Leaf Microbiota: Plant–Microbe–Microbe Interactions.” Edited by Stanislav Kopriva. *Journal of Experimental Botany* 72 (1): 36–56. https://doi.org/10.1093/jxb/eraa417.

Chen, Shifu, Yanqing Zhou, Yaru Chen, and Jia Gu. 2018. “Fastp: An Ultra-Fast All-in-One FASTQ Preprocessor.” *Bioinformatics* 34 (17): i884–90. https://doi.org/10.1093/bioinformatics/bty560.

Claesson, Marcus J., Qiong Wang, Orla O’Sullivan, Rachel Greene-Diniz, James R. Cole, R. Paul Ross, and Paul W. O’Toole. 2010. “Comparison of Two Next-Generation Sequencing Technologies for Resolving Highly Complex Microbiota Composition Using Tandem Variable 16S rRNA Gene Regions.” *Nucleic Acids Research* 38 (22): e200–e200. https://doi.org/10.1093/nar/gkq873.

Cohan, Frederick M. 2002. “What Are Bacterial Species?” *Annual Review of Microbiology* 56 (1): 457–87. https://doi.org/10.1146/annurev.micro.56.012302.160634.

Coker, Joanna, Kateryna Zhalnina, Clarisse Marotz, Deepan Thiruppathy, Megan Tjuanta, Gavin D’Elia, Rodas Hailu, et al. 2022. “A Reproducible and Tunable Synthetic Soil Microbial Community Provides New Insights into Microbial Ecology.” Edited by Ryan McClure. *mSystems* 7 (6): e00951-22. https://doi.org/10.1128/msystems.00951-22.

Coleman-Derr, Devin, Damaris Desgarennes, Citlali Fonseca-Garcia, Stephen Gross, Scott Clingenpeel, Tanja Woyke, Gretchen North, Axel Visel, Laila P. Partida-Martinez, and

Susannah G. Tringe. 2016. “Plant Compartment and Biogeography Affect Microbiome Composition in Cultivated and Native Agave Species.” *New Phytologist* 209 (2): 798–811.

Crandall, Sharifa G., Kaitlin M. Gold, María Del Mar Jiménez-Gasco, Camila C. Filgueiras, and Denis S. Willett. 2020. “A Multi-Omics Approach to Solving Problems in Plant Disease Ecology.” Edited by Sabrina Sarrocco. *PLOS ONE* 15 (9): e0237975. https://doi.org/10.1371/journal.pone.0237975.

Dahlstrom, Kurt M., and Dianne K. Newman. 2022. “Soil Bacteria Protect Fungi from Phenazines by Acting as Toxin Sponges.” *Current Biology* 32 (2): 275-288.e5. https://doi.org/10.1016/j.cub.2021.11.002.

Danecek, Petr, James K Bonfield, Jennifer Liddle, John Marshall, Valeriu Ohan, Martin O Pollard, Andrew Whitwham, et al. 2021. “Twelve Years of SAMtools and BCFtools.” *GigaScience* 10 (2): giab008. https://doi.org/10.1093/gigascience/giab008.

Daniel, Rolf. 2005. “The Metagenomics of Soil.” *Nature Reviews Microbiology* 3 (6): 470–78. https://doi.org/10.1038/nrmicro1160.

Davis, Kathryn E. R., Shayne J. Joseph, and Peter H. Janssen. 2005. “Effects of Growth Medium, Inoculum Size, and Incubation Time on Culturability and Isolation of Soil Bacteria.” *Applied and Environmental Microbiology* 71 (2): 826–34. https://doi.org/10.1128/AEM.71.2.826-834.2005.

D’Costa, Vanessa M., Katherine M. McGrann, Donald W. Hughes, and Gerard D. Wright. 2006. “Sampling the Antibiotic Resistome.” *Science* 311 (5759): 374–77. https://doi.org/10.1126/science.1120800.

De Souza, Rafael Soares Correa, Vagner Katsumi Okura, Jaderson Silveira Leite Armanhi, Beatriz Jorrín, Núria Lozano, Márcio José Da Silva, Manuel González-Guerrero, et al.

2016. “Unlocking the Bacterial and Fungal Communities Assemblages of Sugarcane Microbiome.” *Scientific Reports* 6 (1): 28774. https://doi.org/10.1038/srep28774.

De Weert, Sandra, Hans Vermeiren, Ine H. M. Mulders, Irene Kuiper, Nico Hendrickx, Guido V. Bloemberg, Jos Vanderleyden, René De Mot, and Ben J. J. Lugtenberg. 2002. “Flagella-Driven Chemotaxis Towards Exudate Components Is an Important Trait for Tomato Root Colonization by *Pseudomonas Fluorescens*.” *Molecular Plant-Microbe Interactions* 15 (11): 1173–80. https://doi.org/10.1094/MPMI.2002.15.11.1173.

Delaux, Pierre-Marc, and Sebastian Schornack. 2021. “Plant Evolution Driven by Interactions with Symbiotic and Pathogenic Microbes.” *Science* 371 (6531): eaba6605. https://doi.org/10.1126/science.aba6605.

Delgado-Baquerizo, Manuel, Peter B. Reich, Chanda Trivedi, David J. Eldridge, Sebastián Abades, Fernando D. Alfaro, Felipe Bastida, et al. 2020. “Multiple Elements of Soil Biodiversity Drive Ecosystem Functions across Biomes.” *Nature Ecology & Evolution* 4 (2): 210–20. https://doi.org/10.1038/s41559-019-1084-y.

DeYoe, Hudson, Robert I. Lonard, Frank W. Judd, Richard Stalter, and Ilka Feller. 2020. “Biological Flora of the Tropical and Subtropical Intertidal Zone: Literature Review for *Rhizophora Mangle* L.” *Journal of Coastal Research* 36 (4): 857. https://doi.org/10.2112/JCOASTRES-D-19-00088.1.

Diao, Muhe, Ruben Sinnige, Karsten Kalbitz, Jef Huisman, and Gerard Muyzer. 2017. “Succession of Bacterial Communities in a Seasonally Stratified Lake with an Anoxic and Sulfidic Hypolimnion.” *Frontiers in Microbiology* 8 (December):2511. https://doi.org/10.3389/fmicb.2017.02511.

Dillon, R.J., and V.M. Dillon. 2004. “The Gut Bacteria Of Insects: Nonpathogenic Interactions.” *Annual Review of Entomology* 49 (1): 71–92. https://doi.org/10.1146/annurev.ento.49.061802.123416.

Dini-Andreote, Francisco, and Jos M. Raaijmakers. 2018. “Embracing Community Ecology in Plant Microbiome Research.” *Trends in Plant Science* 23 (6): 467–69. https://doi.org/10.1016/j.tplants.2018.03.013.

Dini-Andreote, Francisco, James C. Stegen, Jan Dirk Van Elsas, and Joana Falcão Salles. 2015. “Disentangling Mechanisms That Mediate the Balance between Stochastic and Deterministic Processes in Microbial Succession.” *Proceedings of the National Academy of Sciences* 112 (11). https://doi.org/10.1073/pnas.1414261112.

Doolittle, W. Ford, and Olga Zhaxybayeva. 2009. “On the Origin of Prokaryotic Species.” *Genome Research* 19 (5): 744–56. https://doi.org/10.1101/gr.086645.108.

Duchateau, Simon, Jérôme Crouzet, Stéphan Dorey, and Aziz Aziz. 2024. “The Plant-Associated Pantoea Spp. as Biocontrol Agents: Mechanisms and Diversity of Bacteria-Produced Metabolites as a Prospective Tool for Plant Protection.” *Biological Control* 188 (January):105441. https://doi.org/10.1016/j.biocontrol.2024.105441.

Dupuy, Lionel X., and Wendy K. Silk. 2016. “Mechanisms of Early Microbial Establishment on Growing Root Surfaces.” *Vadose Zone Journal* 15 (2): 1–13. https://doi.org/10.2136/vzj2015.06.0094.

Dworkin, Martin, and David Gutnick. 2012. “Sergei Winogradsky: A Founder of Modern Microbiology and the First Microbial Ecologist.” *FEMS Microbiology Reviews* 36 (2): 364–79. https://doi.org/10.1111/j.1574-6976.2011.00299.x.

Edwards, Joseph A., Cameron Johnson, Christian Santos-Medellín, Eugene Lurie, Natraj Kumar Podishetty, Srijak Bhatnagar, Jonathan A. Eisen, and Venkatesan Sundaresan. 2015. "Structure, Variation, and Assembly of the Root-Associated Microbiomes of Rice." *Proceedings of the National Academy of Sciences* 112 (8). https://doi.org/10.1073/pnas.1414592112.

Edwards, Joseph A., Christian M. Santos-Medellín, Zachary S. Liechty, Bao Nguyen, Eugene Lurie, Shane Eason, Gregory Phillips, and Venkatesan Sundaresan. 2018. "Compositional Shifts in Root-Associated Bacterial and Archaeal Microbiota Track the Plant Life Cycle in Field-Grown Rice." Edited by Jeff Gore. *PLOS Biology* 16 (2): e2003862. https://doi.org/10.1371/journal.pbio.2003862.

Ernebjerg, Morten, and Roy Kishony. 2012. "Distinct Growth Strategies of Soil Bacteria as Revealed by Large-Scale Colony Tracking." *Applied and Environmental Microbiology* 78 (5): 1345–52. https://doi.org/10.1128/AEM.06585-11.

Escobar-Zepeda, Alejandra, Arturo Vera-Ponce De León, and Alejandro Sanchez-Flores. 2015. "The Road to Metagenomics: From Microbiology to DNA Sequencing Technologies and Bioinformatics." *Frontiers in Genetics* 6 (December). https://doi.org/10.3389/fgene.2015.00348.

Fan, Miao-Chun, Li-Jun Nan, Ya-Min Zhu, Wei-Min Chen, Ge-Hong Wei, and Yan-Bing Lin. 2018. "*Mitsuaria Noduli* Sp. Nov., Isolated from the Root Nodules of Robinia Pseudoacacia in a Lead–Zinc Mine." *International Journal of Systematic and Evolutionary Microbiology* 68 (1): 87–92. https://doi.org/10.1099/ijsem.0.002459.

Field, Katie J., Silvia Pressel, Jeffrey G. Duckett, William R. Rimington, and Martin I. Bidartondo. 2015. “Symbiotic Options for the Conquest of Land.” *Trends in Ecology & Evolution* 30 (8): 477–86. https://doi.org/10.1016/j.tree.2015.05.007.

Fierer, Noah. 2017. “Embracing the Unknown: Disentangling the Complexities of the Soil Microbiome.” *Nature Reviews Microbiology* 15 (10): 579–90. https://doi.org/10.1038/nrmicro.2017.87.

Fierer, Noah, Diana Nemergut, Rob Knight, and Joseph M. Craine. 2010. “Changes through Time: Integrating Microorganisms into the Study of Succession.” *Research in Microbiology* 161 (8): 635–42. https://doi.org/10.1016/j.resmic.2010.06.002.

Fitzpatrick, Connor R., Julia Copeland, Pauline W. Wang, David S. Guttman, Peter M. Kotanen, and Marc T. J. Johnson. 2018. “Assembly and Ecological Function of the Root Microbiome across Angiosperm Plant Species.” *Proceedings of the National Academy of Sciences* 115 (6). https://doi.org/10.1073/pnas.1717617115.

Fonseca-García, Citlali, Devin Coleman-Derr, Etzel Garrido, Axel Visel, Susannah G. Tringe, and Laila P. Partida-Martínez. 2016. “The Cacti Microbiome: Interplay between Habitat-Filtering and Host-Specificity.” *Frontiers in Microbiology* 7. https://doi.org/10.3389/fmicb.2016.00150.

Foster, R.C. 1988. “Microenvironments of Soil Microorganisms.” *Biology and Fertility of Soils* 6 (3). https://doi.org/10.1007/BF00260816.

Fujishige, Nancy A., Neel N. Kapadia, Peter L. De Hoff, and Ann M. Hirsch. 2006. “Investigations of Rhizobium Biofilm Formation: Rhizobia Form Biofilms.” *FEMS Microbiology Ecology* 56 (2): 195–206. https://doi.org/10.1111/j.1574-6941.2005.00044.x.

Gao, Jun-lian, Jing Xue, Hui Yan, Shuai Tong, Mohammad Sayyar Khan, Li-wei Wang, Xiao-jie Mao, Xiuhai Zhang, and Jian-guang Sun. 2019. "*Pantoea Endophytica* Sp. Nov., Novel Endophytic Bacteria Isolated from Maize Planting in Different Geographic Regions of Northern China." *Systematic and Applied Microbiology* 42 (4): 488–94. https://doi.org/10.1016/j.syapm.2019.06.001.

Gao, Jun-lian, Mei Yuan, Xu-ming Wang, Tian-lei Qiu, Ji-wei Li, Hong-can Liu, Xiu-ai Li, Jian Chen, and Jian-guang Sun. 2015. "*Variovorax Guangxiensis* Sp. Nov., an Aerobic, 1-Aminocyclopropane-1-Carboxylate Deaminase Producing Bacterium Isolated from Banana Rhizosphere." *Antonie van Leeuwenhoek* 107 (1): 65–72. https://doi.org/10.1007/s10482-014-0304-3.

Gardan, L., Patrizia Bella, Jean-Marie Meyer, Richard Christen, Philippe Rott, Wafa Achouak, and Régine Samson. 2002. "*Pseudomonas Salomonii* Sp. Nov., Pathogenic on Garlic, and Pseudomonas Palleroniana Sp. Nov., Isolated from Rice." *International Journal of Systematic and Evolutionary Microbiology* 52 (6): 2065–74. https://doi.org/10.1099/ijs.0.02225-0.

Genre, Andrea, Luisa Lanfranco, Silvia Perotto, and Paola Bonfante. 2020. "Unique and Common Traits in Mycorrhizal Symbioses." *Nature Reviews Microbiology* 18 (11): 649–60. https://doi.org/10.1038/s41579-020-0402-3.

Gest, H. 2004. "The Discovery of Microorganisms by Robert Hooke and Antoni van Leeuwenhoek, Fellows of The Royal Society." *Notes and Records: The Royal Society Journal of the History of Science* 58 (2): 187–201.

Gill, Steven R., Mihai Pop, Robert T. DeBoy, Paul B. Eckburg, Peter J. Turnbaugh, Buck S. Samuel, Jeffrey I. Gordon, David A. Relman, Claire M. Fraser-Liggett, and Karen E.

Nelson. 2006. “Metagenomic Analysis of the Human Distal Gut Microbiome.” *Science* 312 (5778): 1355–59. https://doi.org/10.1126/science.1124234.

Goris, Johan, Konstantinos T. Konstantinidis, Joel A. Klappenbach, Tom Coenye, Peter Vandamme, and James M. Tiedje. 2007. “DNA–DNA Hybridization Values and Their Relationship to Whole-Genome Sequence Similarities.” *International Journal of Systematic and Evolutionary Microbiology* 57 (1): 81–91. https://doi.org/10.1099/ijs.0.64483-0.

Grant, Jason R, Eric Enns, Eric Marinier, Arnab Mandal, Emily K Herman, Chih-yu Chen, Morag Graham, Gary Van Domselaar, and Paul Stothard. 2023. “Proksee: In-Depth Characterization and Visualization of Bacterial Genomes.” *Nucleic Acids Research* 51 (W1): W484–92. https://doi.org/10.1093/nar/gkad326.

Großkopf, Tobias, and Orkun S Soyer. 2014. “Synthetic Microbial Communities.” *Current Opinion in Microbiology* 18 (April):72–77. https://doi.org/10.1016/j.mib.2014.02.002.

Haack, Frederike S., Anja Poehlein, Cathrin Kröger, Christian A. Voigt, Meike Piepenbring, Helge B. Bode, Rolf Daniel, Wilhelm Schäfer, and Wolfgang R. Streit. 2016. “Molecular Keys to the *Janthinobacterium* and *Duganella Spp.* Interaction with the Plant Pathogen *Fusarium Graminearum*.” *Frontiers in Microbiology* 7 (October). https://doi.org/10.3389/fmicb.2016.01668.

Habekost, Maike, Nico Eisenhauer, Stefan Scheu, Sibylle Steinbeiss, Alexandra Weigelt, and Gerd Gleixner. 2008. “Seasonal Changes in the Soil Microbial Community in a Grassland Plant Diversity Gradient Four Years after Establishment.” *Soil Biology and Biochemistry* 40 (10): 2588–95. https://doi.org/10.1016/j.soilbio.2008.06.019.

Haichar, Feth El Zahar, Thierry Heulin, Julien P Guyonnet, and Wafa Achouak. 2016. "Stable Isotope Probing of Carbon Flow in the Plant Holobiont." *Current Opinion in Biotechnology* 41 (October):9–13. https://doi.org/10.1016/j.copbio.2016.02.023.

Hamonts, Kelly, Pankaj Trivedi, Anshu Garg, Caroline Janitz, Jasmine Grinyer, Paul Holford, Frederik C. Botha, Ian C. Anderson, and Brajesh K. Singh. 2018. "Field Study Reveals Core Plant Microbiota and Relative Importance of Their Drivers." *Environmental Microbiology* 20 (1): 124–40. https://doi.org/10.1111/1462-2920.14031.

Hanauer, D. I., J. Frederick, B. Fotinakes, and S. A. Strobel. 2012. "Linguistic Analysis of Project Ownership for Undergraduate Research Experiences." Edited by Hannah Sevian. *CBE—Life Sciences Education* 11 (4): 378–85. https://doi.org/10.1187/cbe.12-04-0043.

Herms, Courtney Horn, Rosanna Catherine Hennessy, Frederik Bak, Dorte Bodin Dresbøll, and Mette Haubjerg Nicolaisen. 2022. "Back to Our Roots: Exploring the Role of Root Morphology as a Mediator of Beneficial Plant–Microbe Interactions." *Environmental Microbiology* 24 (8): 3264–72. https://doi.org/10.1111/1462-2920.15926.

Herz, Katharina, Sophie Dietz, Karin Gorzolka, Sylvia Haider, Ute Jandt, Dierk Scheel, and Helge Bruelheide. 2018. "Linking Root Exudates to Functional Plant Traits." Edited by Alexandra Weigelt. *PLOS ONE* 13 (10): e0204128. https://doi.org/10.1371/journal.pone.0204128.

Hitch, Thomas C.A., Lindsay J. Hall, Sarah Kate Walsh, Gabriel E. Leventhal, Emma Slack, Tomas De Wouters, Jens Walter, and Thomas Clavel. 2022. "Microbiome-Based Interventions to Modulate Gut Ecology and the Immune System." *Mucosal Immunology* 15 (6): 1095–1113. https://doi.org/10.1038/s41385-022-00564-1.

Holmes, B., R. J. Owen, A. G. Steigerwalt, and D. J. Brenner. 1984. "*Flavobacterium Gleum*, a New Species Found in Human Clinical Specimens." *International Journal of Systematic Bacteriology* 34 (1): 21–25. https://doi.org/10.1099/00207713-34-1-21.

Hunt, Martin, Nishadi De Silva, Thomas D. Otto, Julian Parkhill, Jacqueline A. Keane, and Simon R. Harris. 2015. "Circlator: Automated Circularization of Genome Assemblies Using Long Sequencing Reads." *Genome Biology* 16 (1): 294. https://doi.org/10.1186/s13059-015-0849-0.

Jaeger, Astrid C. H., Martin Hartmann, Rafaela Feola Conz, Johan Six, and Emily F. Solly. 2024. "Prolonged Water Limitation Shifts the Soil Microbiome from Copiotrophic to Oligotrophic Lifestyles in Scots Pine Mesocosms." *Environmental Microbiology Reports* 16 (1): e13211. https://doi.org/10.1111/1758-2229.13211.

Jain, Chirag, Luis M. Rodriguez-R, Adam M. Phillippy, Konstantinos T. Konstantinidis, and Srinivas Aluru. 2018. "High Throughput ANI Analysis of 90K Prokaryotic Genomes Reveals Clear Species Boundaries." *Nature Communications* 9 (1): 5114. https://doi.org/10.1038/s41467-018-07641-9.

Jansson, Janet K., and Kirsten S. Hofmockel. 2020. "Soil Microbiomes and Climate Change." *Nature Reviews Microbiology* 18 (1): 35–46. https://doi.org/10.1038/s41579-019-0265-7.

Jia, Jiayuan, Xiaoqiang Wang, Peng Deng, Lin Ma, Sonya M. Baird, Xiangdong Li, and Shi-En Lu. 2020. "*Pseudomonas Glycinae* Sp. Nov. Isolated from the Soybean Rhizosphere." *MicrobiologyOpen* 9 (9): e1101. https://doi.org/10.1002/mbo3.1101.

Jing, Jiayi, Paolina Garbeva, Jos M Raaijmakers, and Marnix H Medema. 2024. “Strategies for Tailoring Functional Microbial Synthetic Communities.” *The ISME Journal* 18 (1): wrae049. https://doi.org/10.1093/ismejo/wrae049.

Johansen, Jens E., Svend J. Binnerup, Niels Kroer, and Lars Mølbak. 2005. “*Luteibacter Rhizovicinus* Gen. Nov., Sp. Nov., a Yellow-Pigmented Gammaproteobacterium Isolated from the Rhizosphere of Barley (*Hordeum Vulgare* L.).” *International Journal of Systematic and Evolutionary Microbiology* 55 (6): 2285–91. https://doi.org/10.1099/ijs.0.63497-0.

Johns, Nathan I, Tomasz Blazejewski, Antonio Lc Gomes, and Harris H Wang. 2016. “Principles for Designing Synthetic Microbial Communities.” *Current Opinion in Microbiology* 31 (June):146–53. https://doi.org/10.1016/j.mib.2016.03.010.

Johnson, John L. 1973. “Use of Nucleic-Acid Homologies in the Taxonomy of Anaerobic Bacteria.” *International Journal of Systematic Bacteriology* 23 (4): 308–15.

Johnson, John L., and William B. Whitman. 2014. “Similarity Analysis of DNAs.” In *Methods for General and Molecular Microbiology*, edited by C. A. Reddy, T. J. Beveridge, J. A. Breznak, G. A. Marzluf, T. M. Schmidt, and L. R. Snyder, 624–52. Washington, DC, USA: ASM Press. https://doi.org/10.1128/9781555817497.ch26.

Johnson, N. C., J-H. Graham, and F. A. Smith. 1997. “Functioning of Mycorrhizal Associations along the Mutualism–Parasitism Continuum.” *New Phytologist* 135 (4): 575–85. https://doi.org/10.1046/j.1469-8137.1997.00729.x.

Johnson, Nancy Collins, and James H. Graham. 2013. “The Continuum Concept Remains a Useful Framework for Studying Mycorrhizal Functioning.” *Plant and Soil* 363 (1–2): 411–19. https://doi.org/10.1007/s11104-012-1406-1.

Kabir, Ahmad H., and Jeffrey L. Bennetzen. 2024. “Molecular Insights into the Mutualism That Induces Iron Deficiency Tolerance in Sorghum Inoculated with Trichoderma Harzianum.” *Microbiological Research* 281 (April):127630. https://doi.org/10.1016/j.micres.2024.127630.

Kim, J.-H., K.-Y. Kim, and C.-J. Cha. 2009. “*Flavobacterium Chungangense* Sp. Nov., Isolated from a Freshwater Lake.” *International Journal of Systematic and Evolutionary Microbiology* 59 (7): 1754–58. https://doi.org/10.1099/ijs.0.007955-0.

Kim, MinJi, Su-Kyoung Shin, and Hana Yi. 2020. “*Mucilaginibacter Celer* Sp. Nov. and Aquirhabdus Parva Gen. Nov., Sp. Nov., Isolated from Freshwater.” *International Journal of Systematic and Evolutionary Microbiology* 70 (10): 5479–87. https://doi.org/10.1099/ijsem.0.004437.

Kimkes, Tom E P, and Matthias Heinemann. 2020. “How Bacteria Recognise and Respond to Surface Contact.” *FEMS Microbiology Reviews* 44 (1): 106–22. https://doi.org/10.1093/femsre/fuz029.

Kirk, Jennifer L, Lee A Beaudette, Miranda Hart, Peter Moutoglis, John N Klironomos, Hung Lee, and Jack T Trevors. 2004. “Methods of Studying Soil Microbial Diversity.” *Journal of Microbiological Methods* 58 (2): 169–88. https://doi.org/10.1016/j.mimet.2004.04.006.

Klein, Malin, Justin D. Stewart, Stephanie S. Porter, James T. Weedon, and E. Toby Kiers. 2022. “Evolution of Manipulative Microbial Behaviors in the Rhizosphere.” *Evolutionary Applications* 15 (10): 1521–36. https://doi.org/10.1111/eva.13333.

Knights, Hayley E., Beatriz Jorrin, Timothy L. Haskett, and Philip S. Poole. 2021. "Deciphering Bacterial Mechanisms of Root Colonization." *Environmental Microbiology Reports* 13 (4): 428–44. https://doi.org/10.1111/1758-2229.12934.

Knudsen, Guy R., and Louise-Marie Dandurand. 2007. "Analysis Of Spatial Patterns Of Rhizoplane Colonization." In *The Spatial Distribution of Microbes in the Environment*, 109–33. Dordrecht: Springer.

Konstantinidis, Konstantinos T. 2023. "Sequence-Discrete Species for Prokaryotes and Other Microbes: A Historical Perspective and Pending Issues." *mLife* 2 (4): 341–49.

Konstantinidis, Konstantinos T, Alban Ramette, and James M Tiedje. 2006. "The Bacterial Species Definition in the Genomic Era." *Philosophical Transactions of the Royal Society B: Biological Sciences* 361 (1475): 1929–40. https://doi.org/10.1098/rstb.2006.1920.

Konstantinidis, Konstantinos T, Ramon Rosselló-Móra, and Rudolf Amann. 2017. "Uncultivated Microbes in Need of Their Own Taxonomy." *The ISME Journal* 11 (11): 2399–2406. https://doi.org/10.1038/ismej.2017.113.

Konstantinidis, Konstantinos T., and James M. Tiedje. 2005. "Genomic Insights That Advance the Species Definition for Prokaryotes." *Proceedings of the National Academy of Sciences* 102 (7): 2567–72. https://doi.org/10.1073/pnas.0409727102.

Koren, Sergey, Brian P. Walenz, Konstantin Berlin, Jason R. Miller, Nicholas H. Bergman, and Adam M. Phillippy. 2017. "Canu: Scalable and Accurate Long-Read Assembly via Adaptive *k* -Mer Weighting and Repeat Separation." *Genome Research* 27 (5): 722–36. https://doi.org/10.1101/gr.215087.116.

Korenblum, Elisa, Yonghui Dong, Jedrzej Szymanski, Sayantan Panda, Adam Jozwiak, Hassan Massalha, Sagit Meir, Ilana Rogachev, and Asaph Aharoni. 2020. "Rhizosphere

Microbiome Mediates Systemic Root Metabolite Exudation by Root-to-Root Signaling." *Proceedings of the National Academy of Sciences* 117 (7): 3874–83. https://doi.org/10.1073/pnas.1912130117.

Krasteva, Petya V., Jiunn C. N. Fong, Nicholas J. Shikuma, Sinem Beyhan, Marcos V. A. S. Navarro, Fitnat H. Yildiz, and Holger Sondermann. 2010. "*Vibrio Cholerae* VpsT Regulates Matrix Production and Motility by Directly Sensing Cyclic Di-GMP." *Science* 327 (5967): 866–68. https://doi.org/10.1126/science.1181185.

Kumar, Anil, Srijana Mukhia, and Rakshak Kumar. 2022. "Production, Characterisation, and Application of Exopolysaccharide Extracted from a Glacier Bacterium *Mucilaginibacter Sp.* ERMR7:07." *Process Biochemistry* 113 (February):27–36. https://doi.org/10.1016/j.procbio.2021.12.018.

Kuzyakov, Yakov, and Evgenia Blagodatskaya. 2015. "Microbial Hotspots and Hot Moments in Soil: Concept & Review." *Soil Biology and Biochemistry* 83 (April):184–99. https://doi.org/10.1016/j.soilbio.2015.01.025.

Landesman, William J, Zachary B Freedman, and David M Nelson. 2019. "Seasonal, Sub-Seasonal and Diurnal Variation of Soil Bacterial Community Composition in a Temperate Deciduous Forest." *FEMS Microbiology Ecology* 95 (2). https://doi.org/10.1093/femsec/fiz002.

Lapenda, J. C., P. A. Silva, M. C. Vicalvi, K. X. F. R. Sena, and S. C. Nascimento. 2015. "Antimicrobial Activity of Prodigiosin Isolated from *Serratia Marcescens* UFPEDA 398." *World Journal of Microbiology and Biotechnology* 31 (2): 399–406. https://doi.org/10.1007/s11274-014-1793-y.

Lebeis, Sarah L., Sur Herrera Paredes, Derek S. Lundberg, Natalie Breakfield, Jase Gehring, Meredith McDonald, Stephanie Malfatti, et al. 2015. “Salicylic Acid Modulates Colonization of the Root Microbiome by Specific Bacterial Taxa.” *Science* 349 (6250): 860–64. https://doi.org/10.1126/science.aaa8764.

Lee, Imchang, Yeong Ouk Kim, Sang-Cheol Park, and Jongsik Chun. 2016. “OrthoANI: An Improved Algorithm and Software for Calculating Average Nucleotide Identity.” *International Journal of Systematic and Evolutionary Microbiology* 66 (2): 1100–1103. https://doi.org/10.1099/ijsem.0.000760.

Lennon, Jay T., and Stuart E. Jones. 2011. “Microbial Seed Banks: The Ecological and Evolutionary Implications of Dormancy.” *Nature Reviews Microbiology* 9:119–30.

Levy, Asaf, Isai Salas Gonzalez, Maximilian Mittelviefhaus, Scott Clingenpeel, Sur Herrera Paredes, Jiamin Miao, Kunru Wang, et al. 2018. “Genomic Features of Bacterial Adaptation Toplants.” *Nature Genetics* 50 (1): 138–50. https://doi.org/10.1038/s41588-017-0012-9.

Li, Heng, and Richard Durbin. 2009. “Fast and Accurate Short Read Alignment with Burrows–Wheeler Transform.” *Bioinformatics* 25 (14): 1754–60. https://doi.org/10.1093/bioinformatics/btp324.

Ling, Ning, Tingting Wang, and Yakov Kuzyakov. 2022. “Rhizosphere Bacteriome Structure and Functions.” *Nature Communications* 13 (1): 836. https://doi.org/10.1038/s41467-022-28448-9.

Liu, Yong-Xin, Yuan Qin, and Yang Bai. 2019. “Reductionist Synthetic Community Approaches in Root Microbiome Research.” *Current Opinion in Microbiology* 49 (June):97–102. https://doi.org/10.1016/j.mib.2019.10.010.

Lloyd, Karen G, Andrew D Steen, Joshua Ladau, Junqi Yin, and Lonnie Crosby. 2018. "Phylogenetically Novel Uncultured Microbial Cells Dominate Earth Microbiomes." *mSystems* 3 (5).

Lombardino, Jonathan, Swati Bijlani, Nitin K. Singh, Jason M. Wood, Richard Barker, Simon Gilroy, Clay C. C. Wang, and Kasthuri Venkateswaran. 2022. "Genomic Characterization of Potential Plant Growth-Promoting Features of *Sphingomonas* Strains Isolated from the International Space Station." Edited by Kim M. Handley. *Microbiology Spectrum* 10 (1): e01994-21. https://doi.org/10.1128/spectrum.01994-21.

Lu, Huibin, Tongchu Deng, Zhipeng Cai, Feifei Liu, Xunan Yang, Yonghong Wang, and Meiying Xu. 2020. "*Janthinobacterium Violaceinigrum* Sp. Nov., *Janthinobacterium Aquaticum* Sp. Nov. and *Janthinobacterium Rivuli* Sp. Nov., Isolated from a Subtropical Stream in China." *International Journal of Systematic and Evolutionary Microbiology* 70 (4): 2719–25. https://doi.org/10.1099/ijsem.0.004097.

Lu, Huibin, Da Song, Tongchu Deng, Chengfang Mei, and Meiying Xu. 2022. "*Duganella Vulcania* Sp. Nov., *Rugamonas Fusca* Sp. Nov., *Rugamonas Brunnea* Sp. Nov. and *Rugamonas Apoptosis* Sp. Nov., Isolated from Subtropical Streams, and Phylogenomic Analyses of the Genera *Janthinobacterium*, *Duganella*, *Rugamonas*, *Pseudoduganella* and *Massilia*." *International Journal of Systematic and Evolutionary Microbiology* 72 (9). https://doi.org/10.1099/ijsem.0.005407.

Lu, Jennifer, Florian P. Breitwieser, Peter Thielen, and Steven L. Salzberg. 2017. "Bracken: Estimating Species Abundance in Metagenomics Data." *PeerJ Computer Science* 3 (January):e104. https://doi.org/10.7717/peerj-cs.104.

Lu, Jennifer, Natalia Rincon, Derrick E. Wood, Florian P. Breitwieser, Christopher Pockrandt, Ben Langmead, Steven L. Salzberg, and Martin Steinegger. 2022. "Metagenome Analysis Using the Kraken Software Suite." *Nature Protocols* 17 (12): 2815–39. https://doi.org/10.1038/s41596-022-00738-y.

Lundberg, Derek S., Sarah L. Lebeis, Sur Herrera Paredes, Scott Yourstone, Jase Gehring, Stephanie Malfatti, Julien Tremblay, et al. 2012. "Defining the Core Arabidopsis Thaliana Root Microbiome." *Nature* 488 (7409): 86–90. https://doi.org/10.1038/nature11237.

Luo, Chengwei, Seth T. Walk, David M. Gordon, Michael Feldgarden, James M. Tiedje, and Konstantinos T. Konstantinidis. 2011. "Genome Sequencing of Environmental *Escherichia Coli* Expands Understanding of the Ecology and Speciation of the Model Bacterial Species." *Proceedings of the National Academy of Sciences* 108 (17): 7200–7205. https://doi.org/10.1073/pnas.1015622108.

Lv, Luqiong, Jinyan Luo, Temoor Ahmed, Haitham E. M. Zaki, Ye Tian, Muhammad Shafiq Shahid, Jianping Chen, and Bin Li. 2022. "Beneficial Effect and Potential Risk of *Pantoea* on Rice Production." *Plants* 11 (19): 2608. https://doi.org/10.3390/plants11192608.

Martins, Samuel J., Josephine Pasche, Hiago Antonio O. Silva, Gijs Selten, Noah Savastano, Lucas Magalhães Abreu, Harsh P. Bais, et al. 2023. "The Use of Synthetic Microbial Communities to Improve Plant Health." *Phytopathology*® 113 (8): 1369–79. https://doi.org/10.1094/PHYTO-01-23-0016-IA.

Mayjonade, Baptiste, Jérôme Gouzy, Cécile Donnadieu, Nicolas Pouilly, William Marande, Caroline Callot, Nicolas Langlade, and Stéphane Muños. 2016. "Extraction of

High-Molecular-Weight Genomic DNA for Long-Read Sequencing of Single Molecules." *BioTechniques* 61 (4): 203–5. https://doi.org/10.2144/000114460.

McClure, Ryan, Dan Naylor, Yuliya Farris, Michelle Davison, Sarah J. Fansler, Kirsten S. Hofmockel, and Janet K. Jansson. 2020. "Development and Analysis of a Stable, Reduced Complexity Model Soil Microbiome." *Frontiers in Microbiology* 11 (August):1987. https://doi.org/10.3389/fmicb.2020.01987.

Mee, Michael T., James J. Collins, George M. Church, and Harris H. Wang. 2014. "Syntrophic Exchange in Synthetic Microbial Communities." *Proceedings of the National Academy of Sciences* 111 (20). https://doi.org/10.1073/pnas.1405641111.

Meier-Kolthoff, Jan P., and Markus Göker. 2019. "TYGS Is an Automated High-Throughput Platform for State-of-the-Art Genome-Based Taxonomy." *Nature Communications* 10 (1): 2182. https://doi.org/10.1038/s41467-019-10210-3.

Mendes, Rodrigo, Paolina Garbeva, and Jos M. Raaijmakers. 2013. "The Rhizosphere Microbiome: Significance of Plant Beneficial, Plant Pathogenic, and Human Pathogenic Microorganisms." *FEMS Microbiology Reviews* 37 (5): 634–63. https://doi.org/10.1111/1574-6976.12028.

Mendes, Rodrigo, Marco Kruijt, Irene De Bruijn, Ester Dekkers, Menno Van Der Voort, Johannes H. M. Schneider, Yvette M. Piceno, et al. 2011. "Deciphering the Rhizosphere Microbiome for Disease-Suppressive Bacteria." *Science* 332 (6033): 1097–1100. https://doi.org/10.1126/science.1203980.

Miao, Wei, Lirong Song, Sang Ba, Longxian Zhang, Guiquan Guan, Zhang Zhang, and Kang Ning. 2020. "Protist 10,000 Genomes Project." *The Innovation* 1 (3): 100058. https://doi.org/10.1016/j.xinn.2020.100058.

Morales, Sergio E., and William E. Holben. 2011. “Linking Bacterial Identities and Ecosystem Processes: Can ‘Omic’ Analyses Be More than the Sum of Their Parts?: Toward Linking Bacterial Identities and Ecosystem Processes.” *FEMS Microbiology Ecology* 75 (1): 2–16. https://doi.org/10.1111/j.1574-6941.2010.00938.x.

Morales-Ruíz, Leslie-Mariana, Mariana Rodríguez-Cisneros, Jeniffer-Chris Kerber-Díaz, Fernando-Uriel Rojas-Rojas, J. Antonio Ibarra, and Paulina Estrada-de Los Santos. 2022. “*Burkholderia Orbicola* Sp. Nov., a Novel Species within the Burkholderia Cepacia Complex.” *Archives of Microbiology* 204 (3): 178. https://doi.org/10.1007/s00203-022-02778-0.

Mot, Rene, and Jos Vanderleyden. 1991. “Purification of a Root-Adhesive Outer Membrane Protein of Root-Colonizing *Pseudomonas Fluorescens*.” *FEMS Microbiology Letters* 81 (3): 323–27. https://doi.org/10.1111/j.1574-6968.1991.tb04780.x.

Mühle, Estelle, Chloé Abry, Priscilla Leclerc, Gogoa-Marthe Goly, Alexis Criscuolo, Hans-Jürgen Busse, Peter Kämpfer, Jean-François Bernardet, Dominique Clermont, and Olivier Chesneau. 2021. “*Flavobacterium Bizetiae Sp.* Nov., Isolated from Diseased Freshwater Fish in Canada at the End of the 1970s.” *International Journal of Systematic and Evolutionary Microbiology* 71 (1). https://doi.org/10.1099/ijsem.0.004576.

Nadarajah, Kalaivani, and Nur Sabrina Natasha Abdul Rahman. 2021. “Plant–Microbe Interaction: Aboveground to Belowground, from the Good to the Bad.” *International Journal of Molecular Sciences* 22 (19): 10388. https://doi.org/10.3390/ijms221910388.

Neal, Andrew L., Shakoor Ahmad, Ruth Gordon-Weeks, and Jurriaan Ton. 2012. “Benzoxazinoids in Root Exudates of Maize Attract *Pseudomonas Putida* to the

Rhizosphere." Edited by Ching-Hong Yang. *PLoS ONE* 7 (4): e35498. https://doi.org/10.1371/journal.pone.0035498.

Nealson, Kenneth H., and Steven E. Finkel. 2011. "Electron Flow and Biofilms." *MRS Bulletin* 36 (5): 380–84. https://doi.org/10.1557/mrs.2011.69.

Nielsen, Alex T., Tim Tolker-Nielsen, Kim B. Barken, and Søren Molin. 2000. "Role of Commensal Relationships on the Spatial Structure of a Surface-attached Microbial Consortium." *Environmental Microbiology* 2 (1): 59–68. https://doi.org/10.1046/j.1462-2920.2000.00084.x.

Nishida, Akifumi, Mayuko Nakagawa, and Masayuki Yamamura. 2021. "Determinism of Microbial Community Assembly by Drastic Environmental Change." *PLOS One* 16 (12): e0260591. https://doi.org/10.1371/journal.pone.0260591.

Nishu, Susmita Das, Jee Hyun No, and Tae Kwon Lee. 2022. "Transcriptional Response and Plant Growth Promoting Activity of *Pseudomonas Fluorescens* DR397 under Drought Stress Conditions." Edited by Lindsey Price Burbank. *Microbiology Spectrum* 10 (4): e00979-22. https://doi.org/10.1128/spectrum.00979-22.

Niu, Ben, Joseph Nathaniel Paulson, Xiaoqi Zheng, and Roberto Kolter. 2017. "Simplified and Representative Bacterial Community of Maize Roots." *Proceedings of the National Academy of Sciences* 114 (12). https://doi.org/10.1073/pnas.1616148114.

Ochman, Howard, Jeffrey G Lawrence, and Eduardo A Groisman. 2000. "Lateral Gene Transfer and the Nature of Bacterial Innovation." *Nature* 405 (6784): 299–304.

Ofek-Lalzar, Maya, Noa Sela, Milana Goldman-Voronov, Stefan J. Green, Yitzhak Hadar, and Dror Minz. 2014. "Niche and Host-Associated Functional Signatures of the Root

Surface Microbiome." *Nature Communications* 5 (1): 4950. https://doi.org/10.1038/ncomms5950.

Ogunseitan, Oladele. 2005. *Microbial Diversity: Form and Function in Prokaryotes*. Suffolk: Blackwell Science Ltd.

Olanrewaju, Oluwaseyi Samuel, Ayansina Segun Ayangbenro, Bernard R. Glick, and Olubukola Oluranti Babalola. 2019. "Plant Health: Feedback Effect of Root Exudates-Rhizobiome Interactions." *Applied Microbiology and Biotechnology* 103 (3): 1155–66. https://doi.org/10.1007/s00253-018-9556-6.

Pace, Norman R. 1997. "A Molecular View of Microbial Diversity and the Biosphere." *Science* 276 (5313): 734–40. https://doi.org/10.1126/science.276.5313.734.

Page, Andrew J., Carla A. Cummins, Martin Hunt, Vanessa K. Wong, Sandra Reuter, Matthew T.G. Holden, Maria Fookes, Daniel Falush, Jacqueline A. Keane, and Julian Parkhill. 2015. "Roary: Rapid Large-Scale Prokaryote Pan Genome Analysis." *Bioinformatics* 31 (22): 3691–93. https://doi.org/10.1093/bioinformatics/btv421.

Pang, Fei, Qing Li, Manoj Kumar Solanki, Zhen Wang, Yong-Xiu Xing, and Deng-Feng Dong. 2024. "Soil Phosphorus Transformation and Plant Uptake Driven by Phosphate-Solubilizing Microorganisms." *Frontiers in Microbiology* 15 (March):1383813. https://doi.org/10.3389/fmicb.2024.1383813.

Pankievicz, Vânia Carla Silva, Pierre-Marc Delaux, Valentina Infante, Hayley H. Hirsch, Shanmugam Rajasekar, Pablo Zamora, Dhileepkumar Jayaraman, Claudia Irene Calderon, Alan Bennett, and Jean-Michel Ané. 2022. "Nitrogen Fixation and Mucilage Production on Maize Aerial Roots Is Controlled by Aerial Root Development and Border Cell

Functions." *Frontiers in Plant Science* 13 (October):977056. https://doi.org/10.3389/fpls.2022.977056.

Patterson, Scott D., and Ruedi H. Aebersold. 2003. "Proteomics: The First Decade and Beyond." *Nature Genetics* 33 (1): 311–23.

Parks, Donovan H., Maria Chuvochina, Pierre-Alain Chaumeil, Christian Rinke, Aaron J. Mussig, and Philip Hugenholtz. 2020. "A Complete Domain-to-Species Taxonomy for Bacteria and Archaea." *Nature Biotechnology* 38 (9): 1079–86. https://doi.org/10.1038/s41587-020-0501-8.

Paul, E. A., and F. E. Clark. 1989. *Soil Microbiology and Biochemistry*. San Diego: Academic Press.

Peiffer, Jason A., Aymé Spor, Omry Koren, Zhao Jin, Susannah Green Tringe, Jeffery L. Dangl, Edward S. Buckler, and Ruth E. Ley. 2013. "Diversity and Heritability of the Maize Rhizosphere Microbiome under Field Conditions." *Proceedings of the National Academy of Sciences* 110 (16): 6548–53. https://doi.org/10.1073/pnas.1302837110.

Peng, Shunqiao, Yiyuan Ren, Ting Yao, Honglong Chu, Yong Gao, Xuelian Tian, and Yanan Zhang. 2023. "First Report of *Pseudomonas Palleroniana* Causing Potato Soft Rot in China." *Plant Disease* 107 (2): 553. https://doi.org/10.1094/PDIS-04-22-0816-PDN.

Peng, Xinhua, Rainer Horn, and Paul Hallett. 2015. "Soil Structure and Its Functions in Ecosystems: Phase Matter & Scale Matter." *Soil and Tillage Research* 146 (March):1–3. https://doi.org/10.1016/j.still.2014.10.017.

Petrushin, Ivan S., Sergei I. Belikov, and Lubov I. Chernogor. 2020. "Draft Genome Sequence of *Flavobacterium* Sp. Strain SLB02, Isolated from the Diseased Sponge

Lubomirskia Baicalensis." Edited by Frank J. Stewart. *Microbiology Resource Announcements* 9 (26): e00530-20. https://doi.org/10.1128/MRA.00530-20.

Pham, Van H T, and Jaisoo Kim. 2016. "Improvement for Isolation of Soil Bacteria by Using Common Culture Media." *Journal Of Pure And Applied Microbiology* 10 (1): 49–59.

Pham, Van H.T., and Jaisoo Kim. 2012. "Cultivation of Unculturable Soil Bacteria." *Trends in Biotechnology* 30 (9): 475–84. https://doi.org/10.1016/j.tibtech.2012.05.007.

Philippot, Laurent, Jos M. Raaijmakers, Philippe Lemanceau, and Wim H. Van Der Putten. 2013. "Going Back to the Roots: The Microbial Ecology of the Rhizosphere." *Nature Reviews Microbiology* 11 (11): 789–99. https://doi.org/10.1038/nrmicro3109.

Poli, Noémie, Christoph Joseph Keel, and Daniel Garrido-Sanz. 2024. "Expanding the Pseudomonas Diversity of the Wheat Rhizosphere: Four Novel Species Antagonizing Fungal Phytopathogens and with Plant-Beneficial Properties." *Frontiers in Microbiology* 15 (July):1440341. https://doi.org/10.3389/fmicb.2024.1440341.

Preston, Gail M. 2004. "Plant Perceptions of Plant Growth-Promoting *Pseudomonas*." *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* 359 (1446): 907–18. https://doi.org/10.1098/rstb.2003.1384.

Pruitt, K. D. 2004. "NCBI Reference Sequence (RefSeq): A Curated Non-Redundant Sequence Database of Genomes, Transcripts and Proteins." *Nucleic Acids Research* 33 (Database issue): D501–4. https://doi.org/10.1093/nar/gki025.

Pühler, Alfred, Matthieu Arlat, Anke Becker, Michael Göttfert, John P Morrissey, and Fergal O'Gara. 2004. "What Can Bacterial Genome Research Teach Us about Bacteria–

Plant Interactions?" *Current Opinion in Plant Biology* 7 (2): 137–47. https://doi.org/10.1016/j.pbi.2004.01.009.

Quince, Christopher, Thomas P Curtis, and William T Sloan. 2008. "The Rational Exploration of Microbial Diversity." *The ISME Journal* 2 (10): 997–1006. https://doi.org/10.1038/ismej.2008.69.

Rabot, E., M. Wiesmeier, S. Schlüter, and H.-J. Vogel. 2018. "Soil Structure as an Indicator of Soil Functions: A Review." *Geoderma* 314 (March):122–37. https://doi.org/10.1016/j.geoderma.2017.11.009.

Ramey, Bronwyn E, Maria Koutsoudis, Susanne B Von Bodman, and Clay Fuqua. 2004. "Biofilm Formation in Plant–Microbe Associations." *Current Opinion in Microbiology* 7 (6): 602–9. https://doi.org/10.1016/j.mib.2004.10.014.

Ramirez, Kelly S., Jonathan W. Leff, Albert Barberán, Scott Thomas Bates, Jason Betley, Thomas W. Crowther, Eugene F. Kelly, et al. 2014. "Biogeographic Patterns in Below-Ground Diversity in New York City's Central Park Are Similar to Those Observed Globally." *Proceedings of the Royal Society B: Biological Sciences* 281 (1795): 20141988. https://doi.org/10.1098/rspb.2014.1988.

Rappé, Michael S., and Stephen J. Giovannoni. 2003. "The Uncultured Microbial Majority." *Annual Review of Microbiology* 57 (1): 369–94. https://doi.org/10.1146/annurev.micro.57.030502.090759.

Ravin, A. W. 1960. "The Origin of Bacterial Species: Genetic Recombina-Tion and Factors Limiting It between Bacterial Populations." *Bacteriological Review* 24 (2).

Reinhold-Hurek, Barbara, and Thomas Hurek. 2011. “Living inside Plants: Bacterial Endophytes.” *Current Opinion in Plant Biology* 14 (4): 435–43. https://doi.org/10.1016/j.pbi.2011.04.004.

Richter-Heitmann, Tim, Thilo Eickhorst, Stefan Knauth, Michael W. Friedrich, and Hannes Schmidt. 2016. “Evaluation of Strategies to Separate Root-Associated Microbial Communities: A Crucial Choice in Rhizobiome Research.” *Frontiers in Microbiology* 7 (May). https://doi.org/10.3389/fmicb.2016.00773.

Riley, Margaret A., and Michelle Lizotte-Waniewski. 2009. “Population Genomics and the Bacterial Species Concept.” In *Horizontal Gene Transfer*, edited by Maria Boekels Gogarten, Johann Peter Gogarten, and Lorraine C. Olendzenski, 532:367–77. Methods in Molecular Biology. Totowa, NJ: Humana Press. https://doi.org/10.1007/978-1-60327-853-9_21.

Roberson, Emily B., and Mary K. Firestone. 1992. “Relationship between Desiccation and Exopolysaccharide Production in a Soil *Pseudomonas* Sp.” *Applied and Environmental Microbiology* 58 (4): 1284–91. https://doi.org/10.1128/aem.58.4.1284-1291.1992.

Rodriguez-Navarro, Dulce N., Marta S. Dardanelli, and Joao E. Ruiz-Sainz. 2007. “Attachment of Bacteria to the Roots of Higher Plants.” *FEMS Microbiology Letters* 272 (2): 127–36. https://doi.org/10.1111/j.1574-6968.2007.00761.x.

Rodriguez-R, Luis M., Roth E. Conrad, Tomeu Viver, Dorian J. Feistel, Blake G. Lindner, Stephanus N. Venter, Luis H. Orellana, Rudolf Amann, Ramon Rossello-Mora, and Konstantinos T. Konstantinidis. 2024. “An ANI Gap within Bacterial Species That Advances the Definitions of Intra-Species Units.” Edited by Igor B. Jouline. *mBio* 15 (1). https://doi.org/10.1128/mbio.02696-23.

Rosenberg, Eugene, Edward F. DeLong, Stephen Lory, Erko Stackebrandt, and Fabiano Thompson, eds. 2013. *The Prokaryotes: Prokaryotic Biology and Symbiotic Associations*. Berlin, Heidelberg: Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-30194-0.

Rosselló-Móra, Ramon, and Rudolf Amann. 2015. "Past and Future Species Definitions for Bacteria and Archaea." *Systematic and Applied Microbiology* 38 (4): 209–16. https://doi.org/10.1016/j.syapm.2015.02.001.

Ryan, Peter R., Emmanuel Delhaize, Michelle Watt, and Alan E. Richardson. 2016. "Plant Roots: Understanding Structure and Function in an Ocean of Complexity." *Annals of Botany* 118 (4): 555–59. https://doi.org/10.1093/aob/mcw192.

Sasse, Joelle, Enrico Martinoia, and Trent Northen. 2018. "Feed Your Friends: Do Plant Exudates Shape the Root Microbiome?" *Trends in Plant Science* 23 (1): 25–41. https://doi.org/10.1016/j.tplants.2017.09.003.

Scales, Brittan S., Robert P. Dickson, John J. LiPuma, and Gary B. Huffnagle. 2014. "Microbiology, Genomics, and Clinical Significance of the *Pseudomonas Fluorescens* Species Complex, an Unappreciated Colonizer of Humans." *Clinical Microbiology Reviews* 27 (4): 927–48. https://doi.org/10.1128/CMR.00044-14.

Schmidt, Hannes, Naoise Nunan, Alexander Höck, Thilo Eickhorst, Christina Kaiser, Dagmar Woebken, and Xavier Raynaud. 2018. "Recognizing Patterns: Spatial Analysis of Observed Microbial Colonization on Root Surfaces." *Frontiers in Environmental Science* 6 (July):61. https://doi.org/10.3389/fenvs.2018.00061.

Schoch, Conrad L., Keith A. Seifert, Sabine Huhndorf, Vincent Robert, John L. Spouge, C. André Levesque, Wen Chen, et al. 2012. "Nuclear Ribosomal Internal Transcribed Spacer

(ITS) Region as a Universal DNA Barcode Marker for *Fungi*." *Proceedings of the National Academy of Sciences* 109 (16): 6241–46. https://doi.org/10.1073/pnas.1117018109.

Schreiter, Susanne, Guo-Chun Ding, Holger Heuer, GÃ¼nter Neumann, Martin Sandmann, Rita Grosch, Siegfried Kropf, and Kornelia Smalla. 2014. "Effect of the Soil Type on the Microbiome in the Rhizosphere of Field-Grown Lettuce." *Frontiers in Microbiology* 5 (April). https://doi.org/10.3389/fmicb.2014.00144.

Seemann, Torsten. 2014. "Prokka: Rapid Prokaryotic Genome Annotation." *Bioinformatics* 30 (14): 2068–69. https://doi.org/10.1093/bioinformatics/btu153.

Selim, Samy, Nosheen Akhtar, Nashwa Hagagy, Awadh Alanazi, Mona Warrad, Eman El Azab, Mohammed Yagoub Mohammed Elamir, et al. 2022. "Selection of Newly Identified Growth-Promoting Archaea Haloferax Species With a Potential Action on Cobalt Resistance in Maize Plants." *Frontiers in Plant Science* 13 (May):872654. https://doi.org/10.3389/fpls.2022.872654.

Seo, Hyojun, Ju Hui Kim, Sang-Moo Lee, and Seon-Woo Lee. 2024. "The Plant-Associated Flavobacterium: A Hidden Helper for Improving Plant Health." *The Plant Pathology Journal* 40 (3): 251–60. https://doi.org/10.5423/PPJ.RW.01.2024.0019.

Shade, Ashley, Clifford S. Hogan, Amy K. Klimowicz, Matthew Linske, Patricia S. McManus, and Jo Handelsman. 2012. "Culturing Captures Members of the Soil Rare Biosphere." *Environmental Microbiology* 14 (9): 2247–52. https://doi.org/10.1111/j.1462-2920.2012.02817.x.

Shakya, Migun, Neil Gottel, Hector Castro, Zamin K. Yang, Lee Gunter, Jessy Labbé, Wellington Muchero, et al. 2013. "A Multifactor Analysis of Fungal and Bacterial

Community Structure in the Root Microbiome of Mature Populus Deltoides Trees." Edited by Vishal Shah. *PLoS ONE* 8 (10): e76382. https://doi.org/10.1371/journal.pone.0076382.

Sharpton, Thomas J. 2014. "An Introduction to the Analysis of Shotgun Metagenomic Data." *Frontiers in Plant Science* 5 (June). https://doi.org/10.3389/fpls.2014.00209.

Shintani, Masaki, Zoe K. Sanchez, and Kazuhide Kimbara. 2015. "Genomics of Microbial Plasmids: Classification and Identification Based on Replication and Transfer Systems and Host Taxonomy." *Frontiers in Microbiology* 6 (March). https://doi.org/10.3389/fmicb.2015.00242.

Shou, Wenying, Sri Ram, and Jose M. G. Vilar. 2007. "Synthetic Cooperation in Engineered Yeast Populations." *Proceedings of the National Academy of Sciences* 104 (6): 1877–82. https://doi.org/10.1073/pnas.0610575104.

Singh, Pratiksha, Prabhat K. Chauhan, Sudhir K. Upadhyay, Rajesh Kumar Singh, Padmanabh Dwivedi, Jing Wang, Devendra Jain, and Mingguo Jiang. 2022. "Mechanistic Insights and Potential Use of Siderophores Producing Microbes in Rhizosphere for Mitigation of Stress in Plants Grown in Degraded Land." *Frontiers in Microbiology* 13 (July):898979. https://doi.org/10.3389/fmicb.2022.898979.

Sokol, Noah W., Eric Slessarev, Gianna L. Marschmann, Alexa Nicolas, Steven J. Blazewicz, Eoin L. Brodie, Mary K. Firestone, et al. 2022. "Life and Death in the Soil Microbiome: How Ecological Processes Influence Biogeochemistry." *Nature Reviews Microbiology* 20 (7): 415–30. https://doi.org/10.1038/s41579-022-00695-z.

Soldan, Riccardo, Francesca Mapelli, Elena Crotti, Sylvia Schnell, Daniele Daffonchio, Ramona Marasco, Marco Fusi, Sara Borin, and Massimiliano Cardinale. 2019. "Bacterial Endophytes of Mangrove Propagules Elicit Early Establishment of the Natural Host and

Promote Growth of Cereal Crops under Salt Stress." *Microbiological Research* 223–225 (June):33–43. https://doi.org/10.1016/j.micres.2019.03.008.

Stewart, Eric J. 2012. "Growing Unculturable Bacteria." *Journal of Bacteriology* 194 (16): 4151–60. https://doi.org/10.1128/JB.00345-12.

Storey, John D., and Robert Tibshirani. 2003. "Statistical Significance for Genomewide Studies." *Proceedings of the National Academy of Sciences* 100 (16): 9440–45. https://doi.org/10.1073/pnas.1530509100.

Strullu-Derrien, Christine, Paul Kenrick, Silvia Pressel, Jeffrey G. Duckett, Jean-Philippe Rioult, and Desire-Georges Strullu. 2014. "Fungal Associations in *Horneophyton Ligneri* from the Rhynie Chert (c. 407 Million Year Old) Closely Resemble Those in Extant Lower Land Plants: Novel Insights into Ancestral Plant–Fungus Symbioses." *New Phytologist* 203 (3): 964–79.

Szteinberg, Gabriela A., and Gabriela C. Weaver. 2013. "Participants' Reflections Two and Three Years after an Introductory Chemistry Course-Embedded Research Experience." *Chem. Educ. Res. Pract.* 14 (1): 23–35. https://doi.org/10.1039/C2RP20115A.

Taffner, Julian, Armin Erlacher, Anastasia Bragina, Christian Berg, Christine Moissl-Eichinger, and Gabriele Berg. 2018. "What Is the Role of Archaea in Plants? New Insights from the Vegetation of Alpine Bogs." *mSphere* 3 (3): e00122-18. https://doi.org/10.1128/mSphere.00122-18.

Tan, Michelle S. F., Aaron P. White, Sadequr Rahman, and Gary A. Dykes. 2016. "Role of Fimbriae, Flagella and Cellulose on the Attachment of *Salmonella Typhimurium* ATCC 14028 to Plant Cell Wall Models." Edited by Michael Hensel. *PLOS One* 11 (6): e0158311. https://doi.org/10.1371/journal.pone.0158311.

Testerman, Todd, Jackie Varga, Molly M. Schiffer, Hailey Donohue, Caroline Vieira Da Silva, and Joerg Graf. 2023. "*Pseudomonas Aphyarum* Sp. Nov., *Pseudomonas Fontis* Sp. Nov., *Pseudomonas Idahonensis* Sp. Nov. and *Pseudomonas Rubra* Sp. Nov., Isolated from in, and around, a Rainbow Trout Farm." *International Journal of Systematic and Evolutionary Microbiology* 73 (12). https://doi.org/10.1099/ijsem.0.006201.

Thomashow, L. S. 1996. "Biological Control of Plant Root Pathogens." *Current Opinion in Biotechnology* 7 (3).

Torsvik, Vigdis, and Lise Øvreås. 2002. "Microbial Diversity and Function in Soil: From Genes to Ecosystems." *Current Opinion in Microbiology* 5 (3): 240–45. https://doi.org/10.1016/S1369-5274(02)00324-7.

Tringe, Susannah Green, Christian Von Mering, Arthur Kobayashi, Asaf A. Salamov, Kevin Chen, Hwai W. Chang, Mircea Podar, et al. 2005. "Comparative Metagenomics of Microbial Communities." *Science* 308 (5721): 554–57. https://doi.org/10.1126/science.1107851.

Trivedi, Pankaj, Jan E. Leach, Susannah G. Tringe, Tongmin Sa, and Brajesh K. Singh. 2020. "Plant–Microbiome Interactions: From Community Assembly to Plant Health." *Nature Reviews Microbiology* 18 (11): 607–21. https://doi.org/10.1038/s41579-020-0412-1.

Turnbull, Amy L., Yibin Liu, and George Lazarovits. 2012. "Isolation of Bacteria from the Rhizosphere and Rhizoplane of Potato (*Solanum Tuberosum*) Grown in Two Distinct Soils Using Semi Selective Media and Characterization of Their Biological Properties." *American Journal of Potato Research* 89 (4): 294–305. https://doi.org/10.1007/s12230-012-9253-4.

Tuson, Hannah H., and Douglas B. Weibel. 2013. “Bacteria–Surface Interactions.” *Soft Matter* 9 (17): 4368. https://doi.org/10.1039/c3sm27705d.

Urón, Paola, Admir J. Giachini, Bernard R. Glick, Márcio J. Rossi, and Francisco X. Nascimento. 2018. “Near-Complete Genome Sequence of *Pseudomonas Palleroniana* MAB3, a Beneficial 1-Aminocyclopropane-1-Carboxylate Deaminase-Producing Bacterium Able To Promote the Growth of Mushrooms and Plants.” *Genome Announcements* 6 (16). https://doi.org/10.1128/genomea.00242-18.

Vandamme, P, B Pot, M Gillis, P De Vos, K Kersters, and J Swings. 1996. “Polyphasic Taxonomy, a Consensus Approach to Bacterial Systematics.” *MICROBIOL. REV.* 60.

Vandenkoornhuyse, Philippe, Achim Quaiser, Marie Duhamel, Amandine Le Van, and Alexis Dufresne. 2015. “The Importance of the Microbiome of the Plant Holobiont.” *New Phytologist* 206 (4): 1196–1206. https://doi.org/10.1111/nph.13312.

van Leeuwen, Pim T, Stanley Brul, Jianbo Zhang, and Meike T Wortel. 2023. “Synthetic Microbial Communities (SynComs) of the Human Gut: Design, Assembly, and Applications.” *FEMS Microbiology Reviews* 47 (2): fuad012. https://doi.org/10.1093/femsre/fuad012.

Venter, J. Craig, Karin Remington, John F. Heidelberg, Aaron L. Halpern, Doug Rusch, Jonathan A. Eisen, Dongying Wu, et al. 2004. “Environmental Genome Shotgun Sequencing of the Sargasso Sea.” *Science* 304 (5667): 66–74. https://doi.org/10.1126/science.1093857.

Viaene, Tom, Sarah Langendries, Stien Beirinckx, Martine Maes, and Sofie Goormachtig. 2016. “*Streptomyces* as a Plant’s Best Friend?” Edited by Gerard Muyzer. *FEMS Microbiology Ecology* 92 (8): fiw119. https://doi.org/10.1093/femsec/fiw119.

Vieira, Selma, Johannes Sikorski, Sophie Dietz, Katharina Herz, Marion Schrumpf, Helge Bruelheide, Dierk Scheel, Michael W Friedrich, and Jörg Overmann. 2020. "Drivers of the Composition of Active Rhizosphere Bacterial Communities in Temperate Grasslands." *The ISME Journal* 14 (2): 463–75. https://doi.org/10.1038/s41396-019-0543-4.

Vitorino, Luciana, and Layara Bessa. 2018. "Microbial Diversity: The Gap between the Estimated and the Known." *Diversity* 10 (2): 46. https://doi.org/10.3390/d10020046.

Vos, Michiel, Alexandra B. Wolf, Sarah J. Jennings, and George A. Kowalchuk. 2013. "Micro-Scale Determinants of Bacterial Diversity in Soil." *FEMS Microbiology Reviews* 37 (6): 936–54. https://doi.org/10.1111/1574-6976.12023.

Walters, William A., Zhao Jin, Nicholas Youngblut, Jason G. Wallace, Jessica Sutter, Wei Zhang, Antonio González-Peña, et al. 2018. "Large-Scale Replicated Field Study of Maize Rhizosphere Identifies Heritable Microbes." *Proceedings of the National Academy of Sciences* 115 (28): 7368–73. https://doi.org/10.1073/pnas.1800918115.

Walterson, Alyssa M., and John Stavrinides. 2015. "*Pantoea:* Insights into a Highly Versatile and Diverse Genus within the Enterobacteriaceae." Edited by Jan Roelof Van Der Meer. *FEMS Microbiology Reviews* 39 (6): 968–84. https://doi.org/10.1093/femsre/fuv027.

Washburne, Alex D., James T. Morton, Jon Sanders, Daniel McDonald, Qiyun Zhu, Angela M. Oliverio, and Rob Knight. 2018. "Methods for Phylogenetic Analysis of Microbiome Data." *Nature Microbiology* 3 (6): 652–61. https://doi.org/10.1038/s41564-018-0156-0.

Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, et al. 1987. "Report of the Ad Hoc Committee on Reconciliation of

Approaches to Bacterial Systematics." *International Journal of Systematic and Evolutionary Microbiology* 37 (4): 463–64. https://doi.org/10.1099/00207713-37-4-463.

Weckwerth, Wolfram. 2003. "Metabolomics in Systems Biology." *Annual Review of Plant Biology* 54 (1): 669–89. https://doi.org/10.1146/annurev.arplant.54.031902.135014.

Wheatley, Rachel M, and Philip S Poole. 2018. "Mechanisms of Bacterial Attachment to Roots." *FEMS Microbiology Reviews*, 42 (4): 448–461.

Wood, Derrick E., Jennifer Lu, and Ben Langmead. 2019. "Improved Metagenomic Analysis with Kraken 2." *Genome Biology* 20 (1): 257. https://doi.org/10.1186/s13059-019-1891-0.

Wu, Ruonan, Gareth Trubl, Neslihan Taş, and Janet K. Jansson. 2022. "Permafrost as a Potential Pathogen Reservoir." *One Earth* 5 (4): 351–60. https://doi.org/10.1016/j.oneear.2022.03.010.

Xia, Qing, Thomas Rufty, and Wei Shi. 2021. "Predominant Microbial Colonizers in the Root Endosphere and Rhizosphere of Turfgrass Systems: *Pseudomonas Veronii*, *Janthinobacterium Lividum*, and *Pseudogymnoascus Spp*." *Frontiers in Microbiology* 12 (March). https://doi.org/10.3389/fmicb.2021.643904.

Xu, Ling, Dan Naylor, Zhaobin Dong, Tuesday Simmons, Grady Pierroz, Kim K. Hixson, Young-Mo Kim, et al. 2018. "Drought Delays Development of the Sorghum Root Microbiome and Enriches for Monoderm Bacteria." *Proceedings of the National Academy of Sciences* 115 (18). https://doi.org/10.1073/pnas.1717308115.

Yagi, Jane M., David Sims, Thomas Brettin, David Bruce, and Eugene L. Madsen. 2009. "The Genome of *Polaromonas Naphthalenivorans* Strain CJ2, Isolated from Coal Tar-contaminated Sediment, Reveals Physiological and Metabolic Versatility and Evolution

through Extensive Horizontal Gene Transfer." *Environmental Microbiology* 11 (9): 2253–70. https://doi.org/10.1111/j.1462-2920.2009.01947.x.

Yang, Suijuan, Xinghai Zhang, Zhaoyun Cao, Kaipeng Zhao, Sai Wang, Mingxue Chen, and Xiufang Hu. 2014. "Growth-Promoting *Sphingomonas Paucimobilis* ZJSH1 Associated with *Dendrobium Officinale* through Phytohormone Production and Nitrogen Fixation." *Microbial Biotechnology* 7 (6): 611–20.

Yin, Chuntao, Juan M. Casa Vargas, Daniel C. Schlatter, Christina H. Hagerty, Scot H. Hulbert, and Timothy C. Paulitz. 2021. "Rhizosphere Community Selection Reveals Bacteria Associated with Reduced Root Disease." *Microbiome* 9 (1). https://doi.org/10.1186/s40168-020-00997-5.

Yin, Chuntao, Christina H. Hagerty, and Timothy C. Paulitz. 2022. "Synthetic Microbial Consortia Derived from Rhizosphere Soil Protect Wheat against a Soilborne Fungal Pathogen." *Frontiers in Microbiology* 13 (August):908981. https://doi.org/10.3389/fmicb.2022.908981.

Young, I. M., and J. W. Crawford. 2004. "Interactions and Self-Organization in the Soil-Microbe Complex." *Science* 304 (5677): 1634–37. https://doi.org/10.1126/science.1097394.

Yu, Ke, Corné M.J. Pieterse, Peter A.H.M. Bakker, and Roeland L. Berendsen. 2019. "Beneficial Microbes Going Underground of Root Immunity." *Plant, Cell & Environment* 42 (10): 2860–70. https://doi.org/10.1111/pce.13632.

Zengler, Karsten, Kirsten Hofmockel, Nitin S. Baliga, Scott W. Behie, Hans C. Bernstein, James B. Brown, José R. Dinneny, et al. 2019. "EcoFABs: Advancing Microbiome Science

through Standardized Fabricated Ecosystems." *Nature Methods* 16 (7): 567–71. https://doi.org/10.1038/s41592-019-0465-0.

Zha, Yuguo, Hui Chong, Pengshuo Yang, and Kang Ning. 2022. "Microbial Dark Matter: From Discovery to Applications." *Genomics, Proteomics & Bioinformatics* 20 (5): 867–81. https://doi.org/10.1016/j.gpb.2022.02.007.

Zhang, Shumeng, Srinivasa R. Chaluvadi, and Jeffrey L. Bennetzen. 2020a. "Draft Genome Sequence of a Serratia Marcescens Strain Isolated from the Pitcher Fluids of a *Sarracenia* Pitcher Plant." Edited by David A. Baltrus. *Microbiology Resource Announcements* 9 (2): e01216-19. https://doi.org/10.1128/MRA.01216-19.

———. 2020b. "Genome Sequence Analysis of *Enterobacter* Sp. C6, Found in the Pitcher Fluids of *Sarracenia Rosea*." Edited by Catherine Putonti. *Microbiology Resource Announcements* 9 (3): e01214-19. https://doi.org/10.1128/MRA.01214-19.

Zhu, Qiyun, Shi Huang, Antonio Gonzalez, Imran McGrath, Daniel McDonald, Niina Haiminen, George Armstrong, et al. 2022. "Phylogeny-Aware Analysis of Metagenome Community Ecology Based on Matched Reference Genomes While Bypassing Taxonomy." Edited by Thomas J. Sharpton. *mSystems* 7 (2): e00167-22. https://doi.org/10.1128/msystems.00167-22.

Zuckerkandl, Emile, and Linus Pauling. 1965. "Molecules as Documents of Evolutionary History." *Journal of Theoretical Biology* 8 (2): 357–66. https://doi.org/10.1016/0022-5193(65)90083-4.