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Characterization and Application of Almond-Derived Bacterial Endophytes

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Quantitative and Systems Biology

by

Jessica Paola Saldierna Guzmán

Committee in charge:
Professor: Stephen C. Hart, Advisor
Professor: Rudy M. Ortiz, Chair
Professor: Clarissa J. Nobile
Professor: Mark J. Sistrom

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The Dissertation of Jessica Paola Saldierna Guzmán is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, Merced
2020

Dedication

I would like to dedicate this thesis to the love of my life, my husband Björn. Thanks to your enormous support, patience and warm hugs during times of struggle, this work was possible.

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Contributing photographer:
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J. Paola Saldierna Guzmán (2011). Identification of Bacteria in Soil Contaminated with High Levels of Cr(VI) by Sequence Analysis of their 16S rRNA. Universidad Autónoma del Estado de Morelos.

J. Paola Saldierna Guzmán (2010). Identification of Bacteria in Soil Contaminated with High Levels of Cr(VI). International Society for Microbial Ecology (ISME).

Outreach

University of California, Merced
DNA extraction workshop for Catheys Valley Elementary School.

Sierra Foothill Charter School
Design and execute experiments to foster children's interest in science.

Lacandon Jungle, Chiapas Mexico
Design activities in a way to motivate children to foster their interest in continuing their education.

Abstract of dissertation

The overall aim of this work was to identify and characterize the endophytic bacteria of almond trees. In order to do this, I first established efficient protocols to remove surface microbes from leaves. My study shows that complete removal of leaf cuticle by the sterilization technique assures loss of epiphytic microbes. Additionally, using electron microscopy, I found that established tests to evaluate surface sterility, PCR and leaf imprints, are unreliable methods to demonstrate plant surface sterility. Applying the inhere established surface sterilization protocols allowed me to analyze the endophytic bacterial composition of three genetically different almond cultivars. My results show that two of these three cultivars were dominated by *Pseudomonadaceae*, while the bacterial composition of the third cultivar consisted mainly of *Streptococcaceae*. Multiple commensal *Streptococcaceae* species are able to suppress growth of pathogenic *Pseudomonadaceae* strains in animals. Therefore, my findings suggest that *Streptococcaceae* endophytes might be useful in the development of strategies for reducing pathogenic impacts of *Pseudomonadaceae* on *Prunus* trees. Additionally, I isolated endophytic bacteria and tested the isolates for plant growth promoting effects. One of the isolates was determined to be a novel *Erwinia gerundensis* strain that I named A4. Application of this bacteria to the roots of the model plant *Arabidopsis thaliana* increases overall *Arabidopsis* biomass and increased root surface. Moreover, inoculation of roots with a transgenic A4 strain expressing reporter genes allowed me to visualize colonization of internal root tissues and subsequently above ground organs. I then sequenced the A4 genome in order to understand its plant growth promoting effects. This revealed that A4's genome encodes for enzymes that synthesize organic acids and siderophores that can provide plants with the essential nutrients phosphorus and iron. Furthermore, A4 has the enzymatic pathway to produce the polyamine spermidine that help plants cope with biotic and abiotic stresses. Altogether, this work not only strongly enhances our knowledge of endophytic bacteria in almonds, but could also serve as a foundation for the development of novel agricultural applications using endophytic microorganisms to improve crop productivity.

1 Introduction

Almonds (*Prunus dulcis*) were introduced to the Americas in the 18th century and today, the USA is the largest producer of this crop (United States Department of Agriculture, 2020a) and exports its almonds to more than 100 countries (United States Department of Agriculture, 2020b). The almond industry has a huge economical value, especially in the Central Valley of California where 80% of the worldwide almond production is located (International Nut and Dried Fruit Council, 2018). The Californian almond industry creates a gross revenue of 21 billion dollars per year and it is responsible for generating one-hundred thousand jobs (Sumner et al., 2015). The Central Valley's top ten producing cultivars in 2019 included Nonpareil, Aldrich and Wood Colony (Almond Board of California, 2019). Nonpareil is the top planted and the most important cultivar due to its high productivity and high market demand (Figure 1-1). About 25,000 ha of almonds were planted in the Central Valley from June 2018 through May 2019, and about 47% of this agricultural land was used for Nonpareil (California Department of Food and Agriculture, 2020). The vast majority of almonds are self-incompatible and, therefore, Nonpareil trees are planted in close proximity to cultivars that bloom during a similar period to ensure fertilization and satisfactory nut yields (Gagnard, 1954; Asai et al., 1996; López et al., 2006; Socias i Company, 2017). Furthermore, almond trees are grafted onto rootstocks for a variety of reasons such as fruit quality, anchorage, pathogen resistance, nutrient uptake, canopy architecture and tolerance to drought and salt stress. Nemaguard is a cross between *Prunus persica* (peach) and *Prunus davidiana* (Chinese wild peach), and is the most widely used rootstock for almond trees (Rubio-Cabetas et al., 2017).

Even though almond cultivation methods have been continuously improved, almond farmers are still facing several problems and plant diseases that result in significant economic losses. Furthermore, usage of fertilizers is a general problem for all crop-producing industries (Savci, 2012). While the insufficient application of fertilizers risk plant health and crop yield, oversupplied nutrients not taken up by

plants may lead to ground- and surface water contamination and can also negatively impact air quality (Savci, 2012). Mitigation of these problems could be achieved through the agricultural use of endophytes.

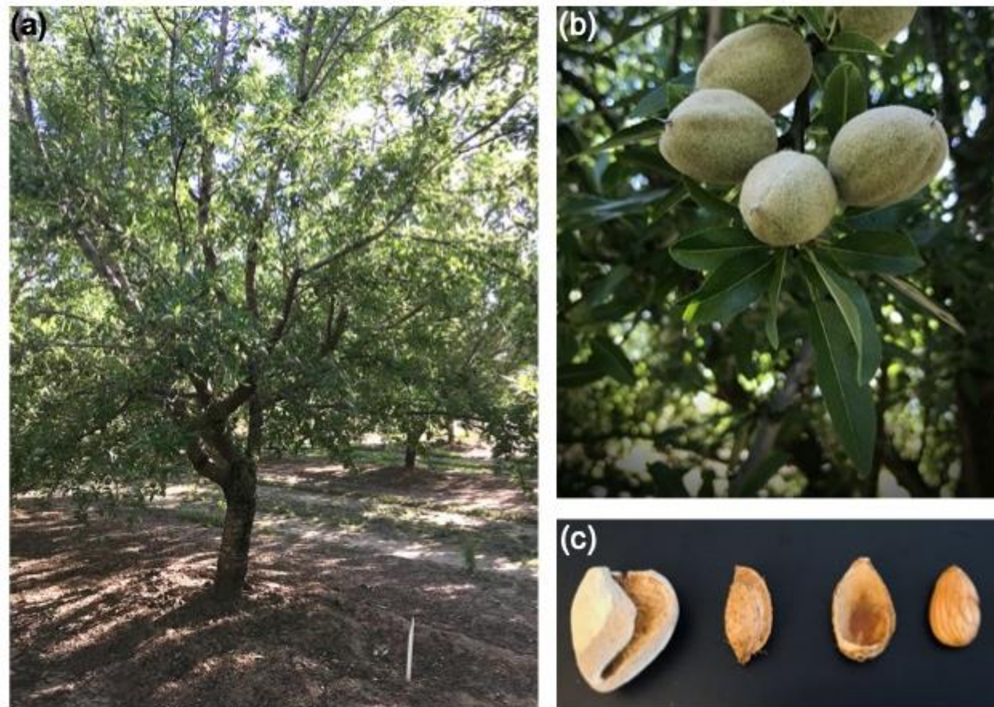

Figure 1-1. Nonpareil cultivar.
(a) Tree growing in the field site used for this study; (b) drupes; (c) ripened drupe, close and open shell and almond seed.

Endophytes have been found living inside all types of plant species and colonizing all of their tissues (Bulgarelli et al., 2013; Frank et al., 2017). They can enter aboveground plant tissues from sources like bioaerosols, precipitation and animals such as pollinators and herbivores (Frank et al., 2017). However, soil is considered to be the most important source of below- and above-ground endophytes, because bacteria can enter the root systems through lesions or emerging lateral roots and once inside the root, they are able to move throughout the plant via vascular systems (Figure 1-2) (Chi et al., 2005; Compant et al., 2010; Hardoim et al., 2015). Endophytes can protect the plant from stresses like phytopathogens and nutrient deficiencies and can help in enhancing growth and yield (Hallmann et al., 1997).

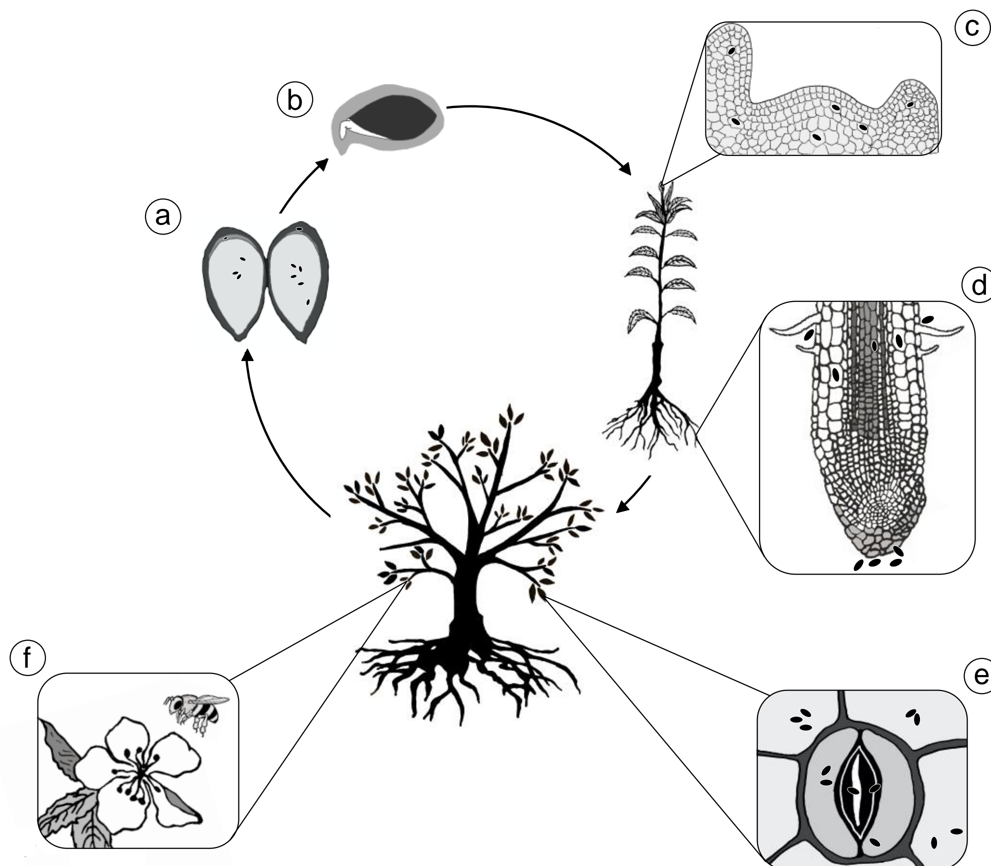

Figure 1-2. Potential colonization routes of bacteria in the life cycle of an almond tree. Colonization via (a) seed, (b) emerging root, (c) shoot apical meristem, (d) root hairs and root tip, (e) stomata, and (f) via insect vectors. Modified from Frank et al., (2017).

One way in which endophytes can enhance nutrient availability to plants is throught the solubilization of insoluble phosphorus (P; Rodríguez and Fraga, 1999). Phosphorus is an essential macronutrient required for plant development and growth, but most of this element exists in soil as an insoluble source; only about 0.1% of the total soil P is directly accessible for plants (Zou et al., 1992). However, several bacteria genera (e.g., *Pseudomonas, Otieno et al., 2015*; *Enterobacter*, Kim et al., 1998; *Erwinia, Goldstein and Liu, 1987*; *Ochrobactrum*, Chakraborty et al., 2009; *Azotobacter*, Sashidhar and Podile, 2009; and *Burkholderia*, Song et al., 2008) have been shown to mobilize poorly accessible P by the production of organic acids. Another essential nutrient for plants is iron (Fe),

which is involved in many important compounds and physiological processes in plants. For example, Fe is involved in the manufacturing process of chlorophyll, and it is required for certain enzymatic functions (Morrissey and Guerinot, 2009). Most of the world's agricultural land is alkaline, and Fe occurs under aerobic conditions mainly as insoluble ferric oxides, hydroxides, and phosphates (Marschner, 1995). As a consequence, these soils do not provide optimal amounts of accessible iron for plant growth (Marschner, 1995). Siderophores, high-affinity iron-chelating compounds that are secreted into the soil (Morrissey and Guerinot, 2009). In contrast to monocotyledonous graminaceous species, dicotyledonous plants are not able to produce phytosiderophores. On the other hand, microbes are able to synthesize siderophores as well and several studies have shown that mono- and dicots are able to access iron by the uptake of bacterial siderophores that provide plants with a source of soluble iron (Bar-Ness et al., 1992; Vansuyt et al., 2007; Jin et al., 2010; ShirleyMatt et al., 2011).

Besides providing their hosts with nutrients, endophytic bacteria can also prevent detrimental effects of phytopathogens by direct antibiosis, competition for space and nutrients or by inducing plant resistance (Khare et al., 2018). For example, several endophytic *Pantoea* strains are able to colonize various crop species such as cocoa, soybean, sugarcane and citrus trees (Torres et al., 2008). Many of these strains have shown antagonistic effects against the bacterium phytopathogen *Erwinia amylovora* by excluding the pathogen from infection sites, production of antibiotics or competing for essential nutrients (Cabrefiga et al., 2011).

Very little is known about the endophytes colonizing almond cultivars and the growth-promoting traits they provide. In order to study almond endophytes, proper removal of surface microbes was essential to avoid erroneous interpretation of subsequent experiments. Therefore, the second chapter focusses on the establishment of efficient surface sterilization methods. This allowed to evaluate the endophytic communities in three of the top producing cultivars (Figure 1-3), which is addressed in the fourth chapter. Furthermore, surface sterilization was

essential to isolate and analyze almond-derived growth promoting endophytes as described in the final chapter of this dissertation.

Figure 1-3. Seeds of the three cultivars analyzed in this study.
Aldrich's shell is semi-soft and well-sealed. Its seed is of medium size and elongated. Nonpareil's is characterized by its soft shell, often refer as "paper shell," and by the sweet taste and medium-sized seed. Wood Colony's shell is medium-hard and well-sealed. It usually contains two seeds, with one seed larger than the other.

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2 Simple Methods to Remove Microbes from Leaf Surfaces

2.1 Abstract

Endophytes have been defined as microorganisms living inside plant tissues without causing negative effects on their hosts. Endophytic microbes have been extensively studied for their plant growth–promoting traits. However, analyses of endophytes require complete removal of epiphytic microorganisms. We found that established tests to evaluate surface sterility, PCR and leaf imprints, are unreliable. Therefore, we used scanning electron microscopy (SEM) as an additional assessment of epiphyte removal. We used a diverse suite of sterilization protocols to remove epiphytic microorganisms from leaves of a gymnosperm and an angiosperm tree to test the influence of leaf morphology on the efficacy of these methods. Additionally, leaf tissue damage was also evaluated by SEM, since damaging the leaves might have an impact on endophytes and could lead to inaccurate assessment of endophytic communities. Our study indicates that complete removal of leaf cuticle by the sterilization technique assures loss of epiphytic microbes and that leaves of different tree species may require different sterilization protocols. Furthermore, our study demonstrates the importance of choosing the appropriate sterilization protocol to prevent erroneous interpretation of host-endophyte interactions. Moreover, it shows the utility of SEM for evaluating the effectiveness of surface sterilization methods and their impact on leaf tissue integrity.

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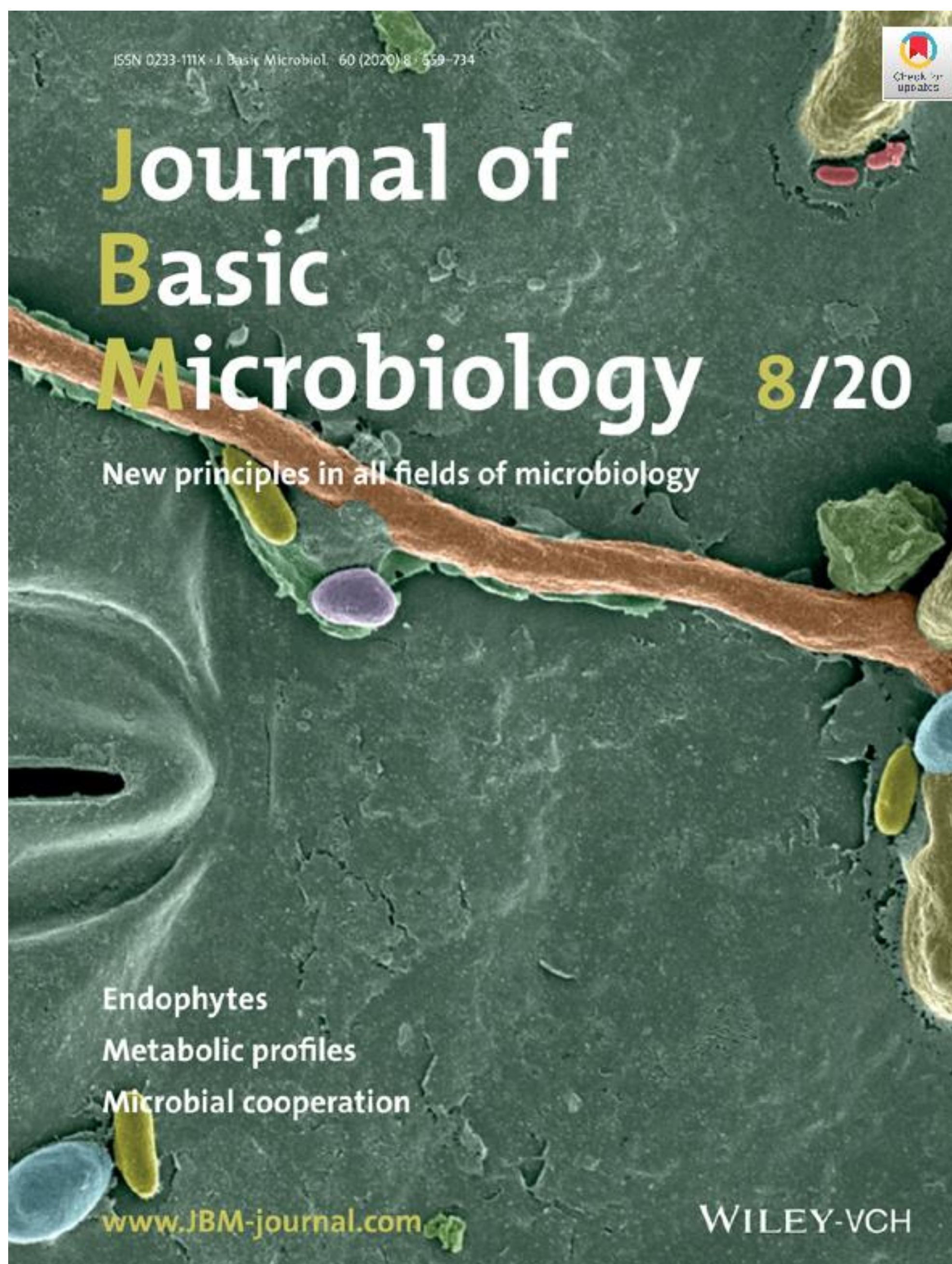

2.2 Cover illustration

Simple Methods to Remove Microbes from Leaf Surfaces
The image shows a leaf surface of cottonwood with microbes after inappropriate removal of epiphytes. This demonstrates the importance of choosing the appropriate sterilization protocol to prevent erroneous interpretation of host-endophyte interactions.

Cover photo: J. Paola Saldierna Guzmán, Kennedy Nguyen and Stephen C. Hart, University of California, Merced, USA

2.3 Introduction

Plant endophytes are described as microorganisms living inside plant tissues without deteriorating their hosts' health (Wilson, 1995; Hallmann et al., 1997; Hardoim et al., 2015). In recent years, studies of plant-endophyte interactions have gained considerable attention due to the potential contributions of these interactions to improve plant health (Li et al., 2019; Durán et al., 2018; Maggini et al., 2017; Tan et al., 2018; Castrillo et al., 2017; Agler et al., 2016). However, several methodological obstacles arise when studying endophytes, including the complete removal of leaf surface microorganisms (i.e., leaf epiphytes). Current methods used to confirm leaf surface sterility generally include polymerase chain reaction (PCR) and leaf prints on nutrient media (Padda et al., 2018; Gao et al., 2015; Carrell et al., 2016; Manter et al., 2010; Ulrich et al., 2008; Shi et al., 2013; Zhao et al., 2016; Carper et al., 2018; Araújo et al., 2002). We included Scanning Electronic Microscopy (SEM) to improve validation of leaf surface sterility and to evaluate potential damage by these treatments on the integrity of leaf tissue. Using SEM, we found that PCR and leaf imprinting results are not always indicative of leaf sterility. Furthermore, our microscopic analyses indicated that different plant species may require different sterilization methods in order to remove all surface microorganisms without significantly damaging leaf tissue.

Here, we describe simple techniques that we have reviewed to remove leaf epiphytic microorganisms. To demonstrate these approaches, we used leaves from two plant species with different phylogenies and leaf morphologies: the gymnosperm tree *Pinus contorta* Douglas ex Loudon var. *murrayana* (Balf.) Engelm. (Sierra lodgepole pine), and the angiosperm tree *Populus fremontti* S. Watson (Fremont cottonwood).

2.4 Methods

2.4.1 Sample collection

Cottonwood leaves were collected at the University of California, Merced, USA (37°22'04.5"N 120°25'21.8"W) and lodgepole pine needles were obtained from Yosemite National Park, CA, USA (37°39'46.9"N, 119°39'38.2"W). The leaves were collected aseptically, placed in sterile bags and immediately transported to the laboratory at the University of California, Merced, where the leaves were surface sterilized using the procedures described below. The effectiveness of four different sterilization methods was evaluated by PCR and leaf imprinting on nutrient medium. Additionally, leaf surfaces were analyzed by SEM.

2.4.2 Application of different surface sterilization protocols

The application of peroxide, ethanol or bleach alone, or combined with each other, are commonly used to remove leaf epiphytic microorganisms (Correa-Galeote et al., 2018; Moyes et al., 2016; Pandey et al., 2016; Peng et al., 2015; Rúa et al., 2016; Sun et al., 2008). Therefore, the efficacy of these reagents for removing leaf epiphytes was evaluated using 1 g of fresh weight leaf tissue for each of the treatments. The four applied sterilization protocols were followed by two rinses with sterile water for 30 seconds and were analyzed in triplicate.

The first treatment consisted of a sonication protocol that was successfully applied to remove epiphytic microbes from *Arabidopsis thaliana* roots. In this procedure, sonication shattered the entire root surface, and no epiphytic microbes were detected (Lundberg et al., 2012). We tested a similar protocol to evaluate the effect on leaf cuticles by applying a frequency of 40 kHz (Branson M1800 Ultrasonic Cleaner, Connecticut, USA) for 10 minutes in 1X Phosphate-Buffered Saline (PBS) solution with 0.02% Silwet L-77 (Lehle Seeds, Texas, USA). In the second treatment, the leaves were washed for 1 minute in 100% ethanol. The third sterilization method consisted of a 5-minute wash with 8.25% sodium hypochlorite (i.e., commercial bleach). The fourth treatment was a 1-minute wash in 30% hydrogen peroxide.

2.4.3 Evaluating surface sterilization protocols

To evaluate the effectiveness of these four treatments to remove leaf epiphytic bacteria by PCR, the last rinse of treated and control leaves (1 g of fresh weight tissue washed in sterile water for 1 minute followed by two 30-second rinses with sterile water) was saved and used for amplification of 16S RNA genes as described previously (Carrell et al., 2016; Moyes et al., 2016), using primers 27f and 1492r (Lane, 1991; Turner et al., 1999). Additionally, treated and non-treated leaves were used to imprint on Lysogeny Broth (LB) media for 30 seconds and incubated at 28 °C for three days (Ren et al., 2019). Overall, a minimum of 20 leaves per treatment and plant species were tested by imprinting.

2.4.4 Electron microscopy

SEM was used to visualize the effectiveness of each sterilization protocol. At least 40 images per treatment and plant species were analyzed. Treated and non-treated leaves were immediately transferred to 2.5% glutaraldehyde in 0.1 M Phosphate-Buffered Saline (PBS) at pH 7.0 for fixation (Dunlap and Adaskaveg, 1997). After 24 hours, samples were washed twice in 0.1 M PBS for 15 minutes. Next, plant tissues were dehydrated through a graded series of 50%, 75%, 95% (in sterile water) and 100% ethanol solutions for 15 minutes for each step. Samples were then transferred to a DCP-1 critical point drying apparatus (Denton Vacuum, New Jersey, USA) using carbon dioxide as the transitional fluid. Afterwards, samples were mounted on aluminum stubs and gold coated with a Polaron SEM Coating Unit E5000 (Bio-Rad, California, USA). A Zeiss Gemini SEM 500 (Carl Zeiss, Baden-Württemberg, Germany) was used for imaging, operated at an accelerating voltage of 3 kV.

2.5 Results

2.5.1 Amplification of 16S RNA genes and imprinting on nutrient media

Polymerase chain reaction (PCR) amplification using the final rinses gave negative results for all samples tested, including the control leaves (Figure 2-1a). Additionally, growth on LB after imprinting was only observed for non-surface sterilized samples and for the ethanol washed samples from cottonwood (Figure 2-1b).

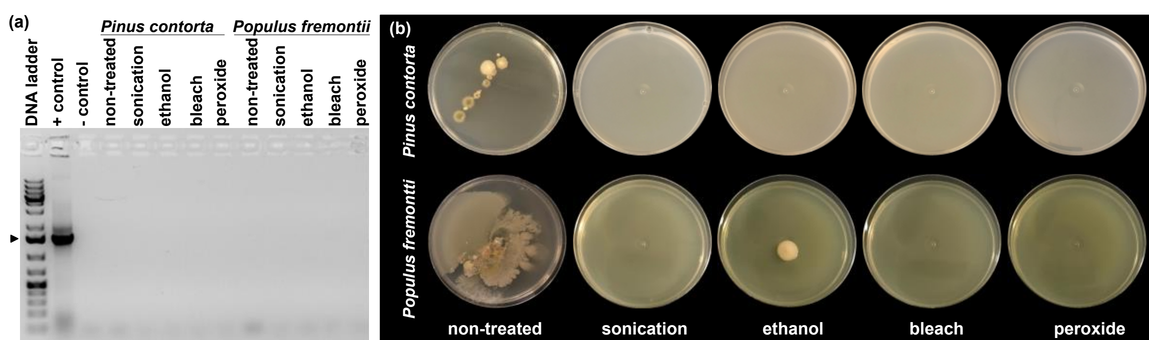

Figure 2-1. Evaluation of leaf surface sterilization protocols.
(a) Amplification of 16S RNA genes. Primers 27f and 1492r were used for amplification. E. coli DNA and water were used as positive and negative controls, respectively. Black arrowhead indicates the 1.5 kb band of the DNA ladder. (b) Non-treated and treated leaves were imprinted in LB media.

2.5.2 Imaging leaf surfaces

Imaging of leaf surfaces using SEM demonstrated that imprinting and PCR are not always reliable methods to evaluate leaf surface sterilization (Figure 2-2). Removal of epiphytic microbes was based on the loss of hyphae or spherical and ellipsoidal structures with the size of bacteria or yeast cells (1 to 10 μm). Untreated samples (Figure 2-2a, b) were compared to the differently treated leaves. Sonicated leaves of both species showed microorganisms remaining on their surfaces (Figure 2-2c, d), indicating that the protocol successfully used for *Arabidopsis* (Lundberg et al., 2012) roots is not suitable for pine and cottonwood leaves. Using ethanol for sterilization was successful in removing surface microbes from lodgepole pine needles (Figure 2-2e), while epiphytes on cottonwood leaves were still present (Figure 2-2f).

In order to test the feasibility of ethanol as a sterilization reagent for cottonwood leaves, the duration of this treatment was extended to 20 minutes. Surface microbes could still be visualized by SEM even after these extended washes (Figure 2-3). For bleach-treated samples, no microbes were observed on leaves of both species (Figure 2-2g, h).

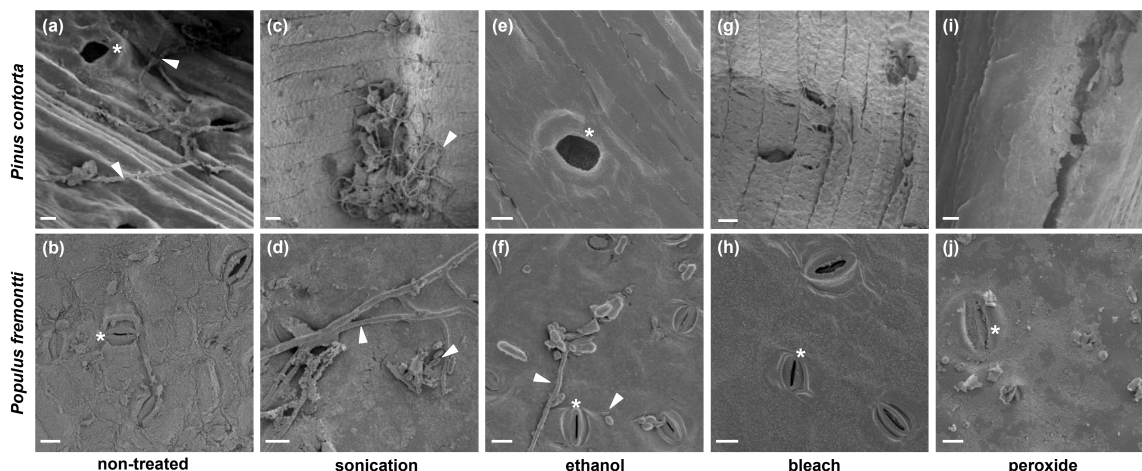

Figure 2-2. Before and after applying the different sterilization protocols.
Leaf surfaces of *Populus fremontti* and *Pinus contorta*. White arrowheads point at examples of epiphytic microbes. Asterisks indicate leaf stomata. Scale bar = 10 μm.

2.5.3 Evaluation of leaf tissue damage

Besides the removal of microbes, we also analyzed leaves for tissue damage. Cottonwood leaves were apparently undamaged by bleach treatments (Figure 2-2h). In contrast, peroxide treatments caused substantial leaf tissue damage, and in most cases, the leaves were in too poor of a condition for mounting to image them. The leaves that survived the treatment were covered with debris that might have originated from damaged leaf tissue (Figure 2-2j). Furthermore, pine needles showed ruptures using bleach and peroxide treatments (Figure 2-2g, i).

2.6 Discussion

Our SEM analyses indicated that removing the cuticle is necessary to achieve leaf surface sterilization, while its partial removal led to the formation of cuticle clusters that contained epiphytic microbes (Figure 2-4). Therefore, we recommend the use of ethanol treatments to achieve proper surface sterilization for lodgepole pine needles and the use of bleach for cottonwood leaves. These respective treatments appeared superior for removing leaf epiphytes because they were effective in removing leaf cuticles without impairing leaf tissue integrity. Additional research is needed to determine, if plant species-specific sterilization treatments are required for effective leaf surface sterilization, or if these two treatments for lodgepole pine needles and Fremont cottonwood leaves are generalizable across other species within their respective gymnosperm and angiosperm groups.

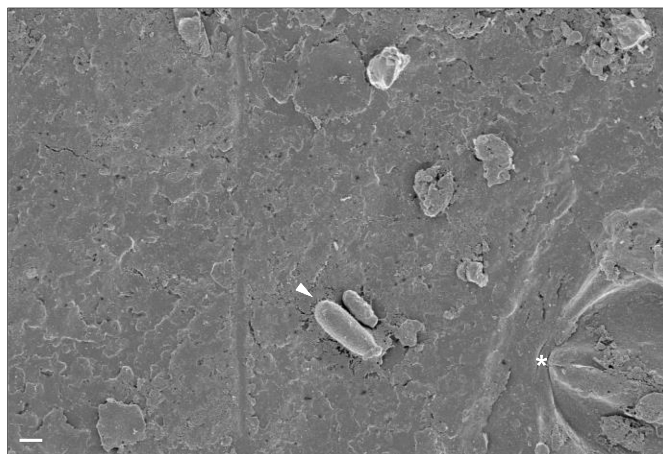

Figure 2-3. After ethanol treatment for 20 minutes.
Leaf surface of *Populus fremontti.* White arrowheads point at an example an epiphytic microbe. The asterisk indicates a leaf stomate. Scale bar = 2 μm.

Taken together, our study reveals that the PCR and leaf imprints may be insufficient to demonstrate leaf surface sterilization, thus leading to inaccurate conclusions about the structure and function of the leaf endophytic communities. Moreover, we show that different plant species may require different treatments to remove leaf epiphytic microbes without causing significant damage to the leaf

tissue; damaging leaf tissue might impact endophytic microbial communities and could lead to erroneous interpretations of microbe-host interactions.

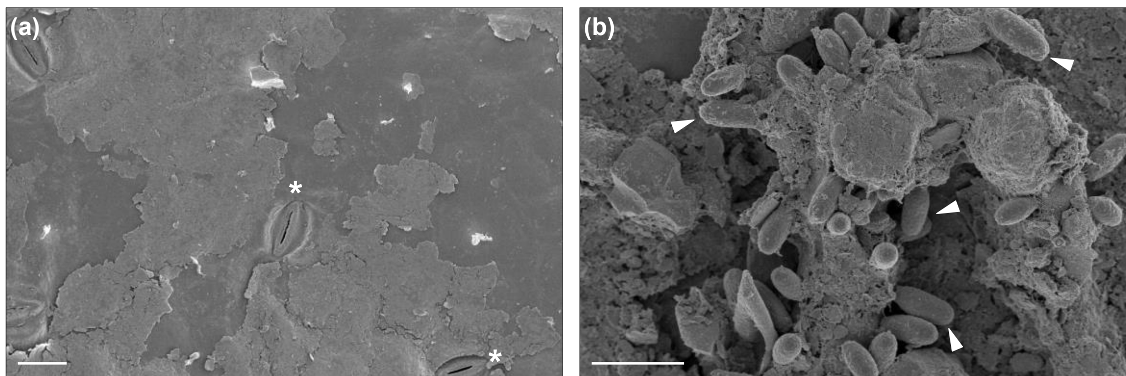

Figure 2-4. After ethanol treatment for 5 minutes.
Leaf surfaces of *Populus fremontti. (a)* Shows partial removal of cuticle and (b) depicts cuticle clusters containing epiphytic microbes. White arrowheads point at examples of epiphytic microbes. Asterisks indicate leaf stomata. Scale bars = 10 μm.

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3 Shoot genotype influences endophytic microbiome of almond cultivars

3.1 Abstract

Almonds (*Prunus dulcis*) are economically important crop trees and are cultivated across an area of 2.1 million ha worldwide. Additionally, they are a globally popular food due to their culinary versatility and beneficial effects on human health. However, almond cultivation is affected by several problems influencing almond productivity and sustainability. Endophytic bacteria colonize inner plant tissues without deteriorating their host's health, and there is ample evidence that many of these endophytes can help plants cope or prevent detrimental effects of biotic and abiotic stresses. To date, little is known about the endophytes colonizing almond trees. In this study, we analyzed the bacterial endophytic communities of three genetically different almond cultivars that were all grafted on the same type of rootstock, growing side by side within a commercial orchard. The experimental set up allowed us to analyze the impact of shoot genotype on endophytes alone, excluding the influence of root genotype, biogeography, and cultivation status. We examined the diversity of leaf bacterial endophytes using cultivation-independent techniques, and assessed the relative abundance of bacterial families. We found that the shoot tissue genotype alone can shape the leaf endophytic community composition of almond trees. This suggests that bacterial colonization itself, or the subsequent establishment inside the leaf, is dependent on the genetic differences between the three almond cultivars analyzed. Two of these three cultivars were dominated by *Pseudomonadaceae*, while the bacterial composition of the third cultivar consisted mainly of *Streptococcaceae*. Because multiple commensal *Streptococcaceae* species are able to suppress growth of pathogenic *Pseudomonadaceae* strains, these findings suggest that *Streptococcaceae* endophytes may be useful in the development of strategies for improving tree productivity and crop yield in almonds and related stone fruits by reducing pathogenic impacts on these trees.

3.2 Introduction

The domestication and breeding history of almonds (*Prunus dulcis*) is not fully described yet, but they likely originated in Southwest Asia and are hypothesized to be the oldest domesticated tree species (Kester and Gradziel, 1996; Atkins et al., 1998; Gradziel, 2011; Zohary et al., 2012). Today, *Prunus dulcis* is an economically important crop tree, cultivated across a 2.1 million ha area worldwide, leading to an annual in-shell production of 3.2 million metric tons (FAOSTAT 2018, www.fao.org/faostat/en/#data/QC). Eighty percent of almond production occurs within the USA, where the almond industry creates a gross revenue of 21 billion dollars per year. Almonds from the USA are exported to more than 100 countries, and are used in many diverse food products around the globe (Sumner et al., 2015; International Nut and Dried Fruit Council, 2018; United States Department of Agriculture, 2020). In addition to their culinary value, almonds have beneficial effects on human health, such as reducing blood pressure, blood sugar, and cholesterol levels (Li et al., 2011; Choudhury et al., 2014; Berryman et al., 2015; Dhillon et al., 2018). All these factors have led to a steadily growing industry, but almond growers are facing several problems influencing the productivity and sustainability of their crop. Besides abiotic factors such as nutrient and water availability, almond production is affected by several diseases; infections caused by *Pseudomonas spp.* result in significant economic losses of almonds, and related stone fruits like cherries (*Prunus yedoensis*), plums (*Prunus subg. Prunus*), nectarines and peaches (*Prunus persica*) (Iacobellis et al., 1990; Mohammadi et al., 2001; Höfte and De Vos, 2007; Kennelly et al., 2007).

Endophytes are described as microorganisms that colonize inner plant tissues for at least part of the microbe's life cycle without deteriorating plant health (Wilson, 1995; Hallmann et al., 1997; Hardoim et al., 2015; Prieto et al., 2017). There is ample evidence that these microbes can help plants cope with abiotic stresses or prevent detrimental effects of phytopathogens (Sturz et al., 2000; Lodewyckx et al., 2002; Ryu et al., 2004; Chen et al., 2010; Yandigeri et al., 2012; Timmusk et al., 2014; Soares et al., 2016; Verma et al., 2018). For instance,

several studies have shown that inoculation of plants with endophytes from arid regions promote drought stress tolerance (Cherif et al., 2015; Niu et al., 2018; Eke et al., 2019). Additionally, studies in *Arabidopsis thaliana* demonstrated that bacteria promote plant health by inhibiting the growth of pathogenic fungi and oomycetes in roots (Durán et al., 2018). Furthermore, a *Pantoea agglomerans* strain isolated from soybean (*Glycine max*) suppressed the growth of the phytopathogenic bacteria, in part, by the production of an antibiotic (Sammer et al., 2012). Taken together, these studies show that the application of endophytic bacteria could be a useful tool to assist almond growers maintain and even improve crop production.

Little is known about the bacterial endophytes colonizing almonds and their potential role in fostering plant stress resistance. Therefore, the primary goal of our study was to investigate and compare the bacterial endophytic communities of three different almond cultivars: Nonpareil, Aldrich, and Wood Colony. Nonpareil is the top planted and the most important almond cultivar due to its high productivity and high market demand (California Department of Food and Agriculture, 2020), but Aldrich and Wood Colony are also among the top ten almond-producing varieties (Almond Board of California, 2019). All three genetically different almond cultivars grew side by side within a commercial orchard. The trees were not only exposed to the same environmental factors, but were also all grafted to rootstocks of the same genotype, Nemaguard. Therefore, our study allowed us to assess the impact of shoot genotype on endophytes alone, excluding the influence of root genotype, biogeography, and cultivation status. We examined the diversity of leaf bacterial endophytes in almond trees using DNA sequencing techniques to infer the relative abundance of bacterial families. Our results may assist in the development of treatments for improving yield and productivity of *Prunus* spp.

3.3 Methods

3.3.1 Sample collection and surface sterilization

Three different almond cultivars (Nonpareil, Aldrich, and Wood Colony) and eight trees per cultivar were analyzed in this study. The rootstock of all three cultivars was Nemaguard, and trees were grown in a commercial orchard located in Modesto, California (37°42'21.8"N, 120°56'55.1"W). Cultivars grew side by side with equal fertilizer inputs and irrigation. They were planted in January 2015, at a spacing of 4.9 m between trees and 6.1 m between rows. On June 2019, leaves of visually healthy trees were collected aseptically, placed in sterile bags, and immediately stored in a portable cooler at 4 °C for transport to the laboratory at University of California, Merced. Leaves were surface sterilized within 3 hours after collection using 8.25% sodium hypochlorite. Removal of surface microbes was confirmed using scanning electron microscopy (Saldierna Guzmán et al., 2020).

3.3.2 Enrichment and DNA extraction

Immediately after surface sterilization, 20 g of fresh surface sterilized leaf tissue was blended for 3 min to enrich bacterial cells, as described previously (Ikeda et al., 2009). Nycodenz was not used because there was only minor accumulation of cell debris. The enriched bacterial cells were frozen using liquid nitrogen and stored at -80 °C for further processing. Total genomic DNA was extracted from the enriched bacterial pellets using the FastDNA SPIN Kit (MP Biomedicals, Irvine, CA, USA), and DNA purification was carried out using the DNA Clean & Concentrator Kit (Zymo, Irvine, CA, USA). Concentration and quality were assessed using gel electrophoresis and a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA). DNA was stored at -20 °C prior to PCR amplification.

3.3.3 PCR amplification

The endophytic bacterial 16S rRNA gene amplification of each sample was conducted using primers 515f (Caporaso et al., 2011) and 1392r (Lane, 1991). PCR reactions were performed in triplicate, each of which included negative and positive controls. The 50 µl PCR reaction contained 1X complete reaction buffer, 200 µM dNTPs, 0.5 µM forward primer, 0.5 µM reverse primer, 1 µM mPNA (KK-GGCAAGTCTTCTTCGGA; PNA Bio, Thousand Oaks, CA, USA), 10 ng of template DNA, and 1.25 units DFS-Taq DNA polymerase (BIORON GmbH, Römerberg, Rheinland-Pfalz, Germany). Cycling conditions were 94 °C for 2 min, followed by 24 cycles of 94 °C for 10 s, 55 °C for 20 s, 72 °C for 1 min, with a final extension of 72 °C for 10 min. A second PCR was performed to remove almond plastid-derived DNA using 799f (Chelius and Triplett, 2001) and 1392r primers, with the same PCR conditions as described above but without the addition of mPNA. PCR products were purified from 1.5% agarose gels using the Zymoclean Gel DNA Recovery Kit (Zymo, Irvine, CA, USA). Amplicons were submitted to GENEWIZ (South Plainfield, NJ, USA) for library construction and sequencing using Illumina MiSeq (2 x 250 bp; Illumina, Inc., San Diego, CA, USA).

3.3.4 Sequence data analysis

The generated raw files were analyzed using the QIIME environment (Caporaso et al., 2010). Sequences that were shorter than 200 bp in length and bases with Q scores lower than 20 were removed from the dataset. Additionally, chimera detection and removal was done using the UCHIME algorithm (Edgar et al., 2011). Sequences were clustered into operational taxonomic units (OTUs) at 97% similarity threshold using VSEARCH, against the Silva 119 database (Rognes et al., 2016). Taxonomic assignment to the Greengenes dataset (DeSantis et al., 2006) was done using the RDP classifier Bayesian algorithm (Wang et al., 2007). Furthermore, reads classified as chloroplast, mitochondria, or singletons were removed from the dataset prior to subsequent analyses. One of the Nonpareil samples was excluded from sequencing due to low concentration after library preparation. In total, 23 samples were used for all subsequent analyses.

3.3.5 Statistical analysis

In order to assess diversity of the observed bacterial taxa, rarefaction depth was equalized to 2,188 reads per sample (Figure 3-1). The species richness estimator Chao 1, as well as Shannon and Simpson diversity indices, were determined using QIIME. One-way ANOVA tests were applied to evaluate significant differences among cultivars using GraphPad Prism 7 (GraphPad Software, La Jolla California, USA). Differences in the community composition of bacterial leaf endophytes among cultivars were assessed by perMANOVA (Anderson, 2001) using the vegan package (Oksanen et al., 2013) in R (R Development Core Team, 2008). Bray-Curtis distances (Bray and Curtis, 1957) were applied to the rarefied data, and Principal Coordinates Analysis (PCoA) was used to visualize these differences. We used an a priori alpha value of 0.05 to denote statistical significance.

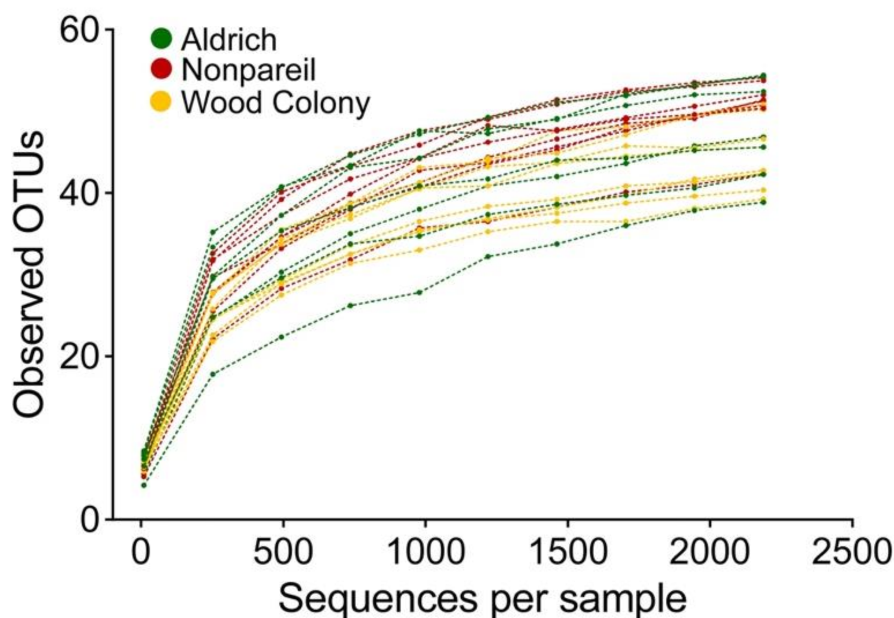

Figure 3-1. Rarefaction curves
Showing the number of operational taxonomic units (OTUs) at different sequencing depths. Based on these curves we equalized sequence depths to 2188 reads for each individual almond tree tested.

3.4 Results

3.4.1 Removal of surface microbes

Because bacteria reside both on the surface and within foliar tissues, it was necessary to remove surface microbes to analyze leaf endophytic bacterial communities of almond cultivars. A bleach treatment that was successfully applied for cottonwood leaves (Saldierna Guzmán et al., 2020) was chosen to remove almond leaf surface microbes due to similarities in leaf morphology between cottonwood and almond tree species. We evaluated the efficacy of the bleach treatment using scanning electron microscopy because our previous analyses showed that imprinting on growth media and PCR are not reliable methods to verify surface sterility (Saldierna Guzmán et al., 2020). Untreated samples showed diverse microorganisms on leaf cuticles (Figure 3-2a), while the bleach treatment led to an apparent complete removal of cuticles, and hence of microbes from leaf surfaces (Figure 3-2b).

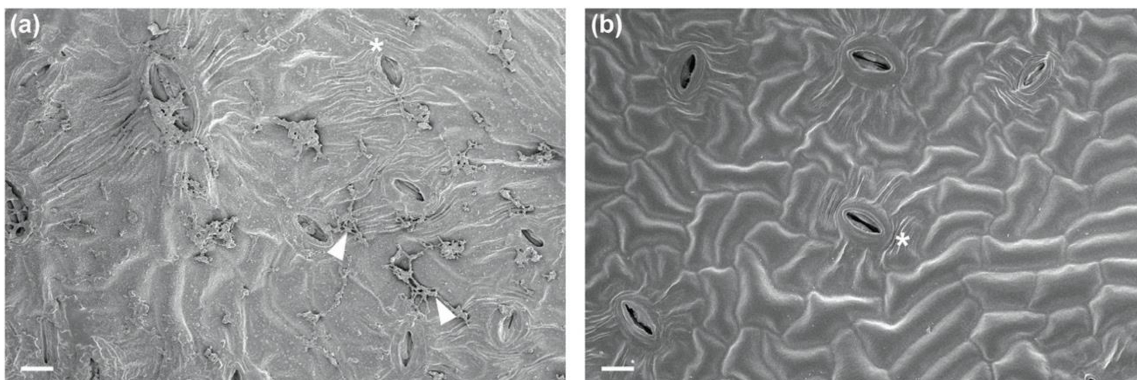

Figure 3-2. Removal of surface microbes of *Prunus dulcis*
Leaf surfaces of *Prunus dulcis* before (a) and after surface sterilization (b). White arrowheads point at examples of epiphytic microbes. Asterisks indicate leaf stomata. Scale bars = 10 μm.

3.4.2 Diversity analysis and community composition

In order to compare samples, rarefaction depth was equalized to 2188 reads per sample (Figure 3-1). Alpha diversity of leaf endophytic communities was measured by Chao1, Shannon, and Simpson indices; no significant differences were found among the three cultivars using one-way ANOVA tests (Chao 1, $p = 0.258$; Shannon, $p = 0.365$; Simpson, $p = 0.625$). This suggests that the number of bacterial OTUs was equal among the three cultivars (Figure 3-3).

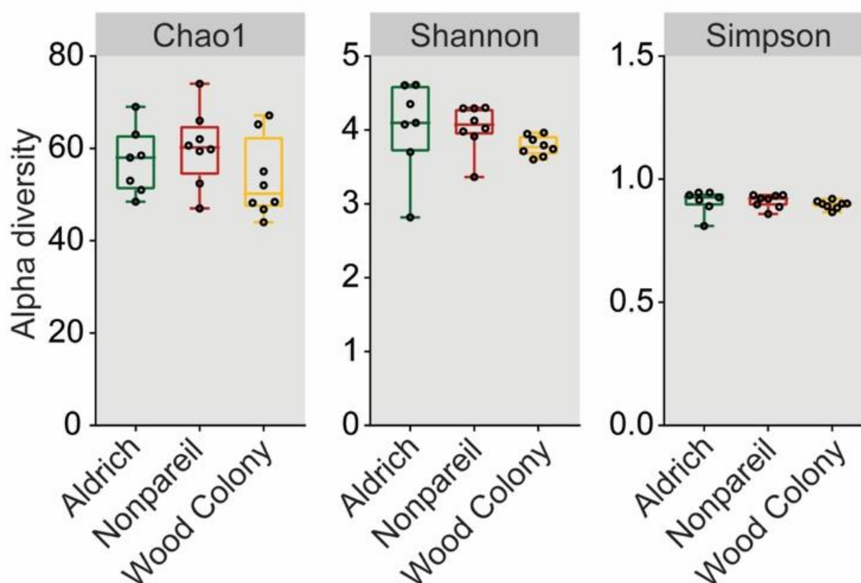

Figure 3-3. Alpha diversity of the almond leaf endophyte communities
Measured by Chao1, Shannon and Simpson indices. All p-values (one-way ANOVA; Chao 1, $p = 0.258$; Shannon, $p = 0.365$; Simpson, $p = 0.625$) exceeded $\alpha = 0.05$, thus no significant differences in alpha diversity were found among the three cultivars.

Despite the lack of detectable differences in alpha diversity (i.e., species richness) of bacteria endophytes, almond cultivar explained 37.6% of the leaf endophytic bacterial composition (Fig. 3-4). Of the three cultivars sampled, leaf endophyte bacteria of Aldrich and Nonpareil cultivars were not significantly different from each other ($p = 0.131$, $R^2 = 0.132$), but Wood Colony's bacteria composition was significantly different from the other two cultivars (Aldrich: $p = 0.002$, $R^2 = 0.284$, Nonpareil: $p < 0.001$, $R^2 = 0.476$).

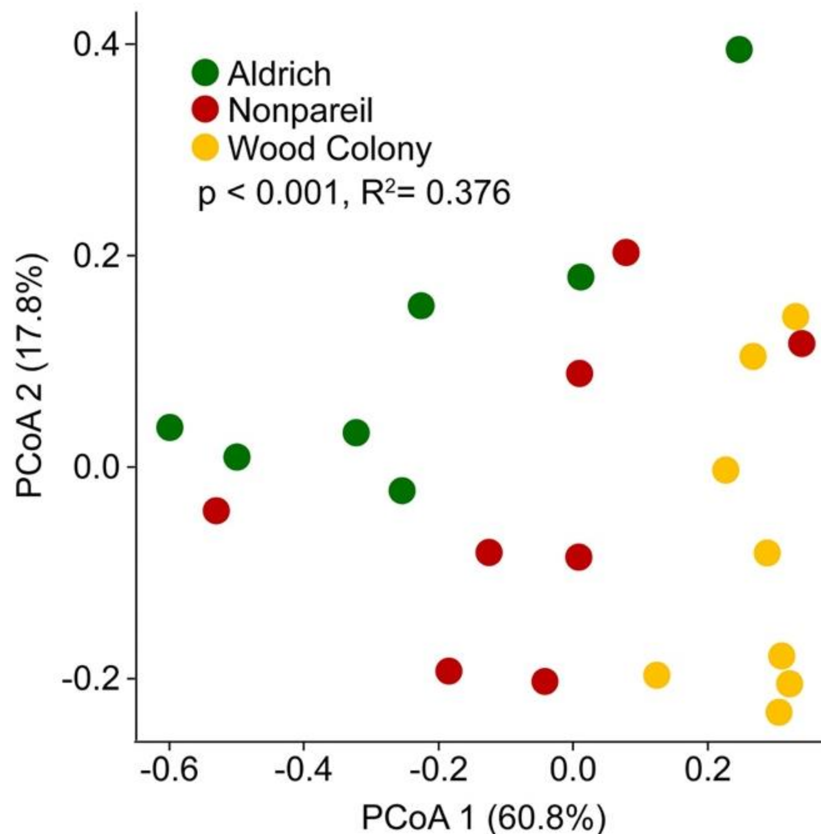

Figure 3-4. Principal coordinate analysis (PCoA) of almond cultivars
Each point represents a leaf bacterial endophyte community from an individual tree. Of the three cultivars analyzed, Wood Colony's leaf bacterial endophytes were significantly different from that of Aldrich and Nonpareil (perMANOVA test, the mean difference is significant at $p < 0.05$). The variance explained by each PCoA axis is given in parentheses.

Of all classified sequences, *Firmicutes* and *Proteobacteria* were the most abundant phyla across all almond cultivars. *Firmicutes* were mainly comprised of *Streptococcaceae* and *Paenibacillaceae* families, while *Proteobacteria* were dominated by *Gammaproteobacteria*, which formed approximately 80% of this phylum across all samples (Figure 3-5). We compared the four most abundant bacterial families among all samples, namely *Enterobacteriaceae* (9.5%), *Oxalobacteraceae* (5.3%), *Pseudomonadaceae* (18.7%) and *Streptococcaceae* (24.2%; Figure 3-6). While the relative abundance of *Enterobacteriaceae* showed no statistical difference among the three almond cultivars, *Oxalobacteraceae* were significantly more abundant in Aldrich than in Wood Colony ($p = 0.044$). Most

strikingly, *Streptococcaceae* and *Pseudomonadaceae* significantly differed among the almond cultivars. On average, *Streptococcaceae* comprised 22.3% of Nonpareil, 5.9% of Aldrich, and 44.4% of Wood Colony leaf endophytes. In contrast, *Pseudomonadaceae* formed 27.3% and 29.4% of leaf bacteria in Nonpareil and Aldrich, but less than 1% in Wood Colony (one-way ANOVA and post hoc Tukey's multiple comparisons tests for *Streptococcaceae*: Aldrich vs. Nonpareil, $p = 0.048$; Nonpareil vs. Wood Colony, $p = 0.013$; Aldrich vs. Wood Colony, $p < 0.001$, and for *Pseudomonadaceae*: Aldrich vs Nonpareil, $p = 0.946$; Nonpareil vs. Wood Colony, $p = 0.002$; Aldrich vs. Wood Colony, $p = 0.001$).

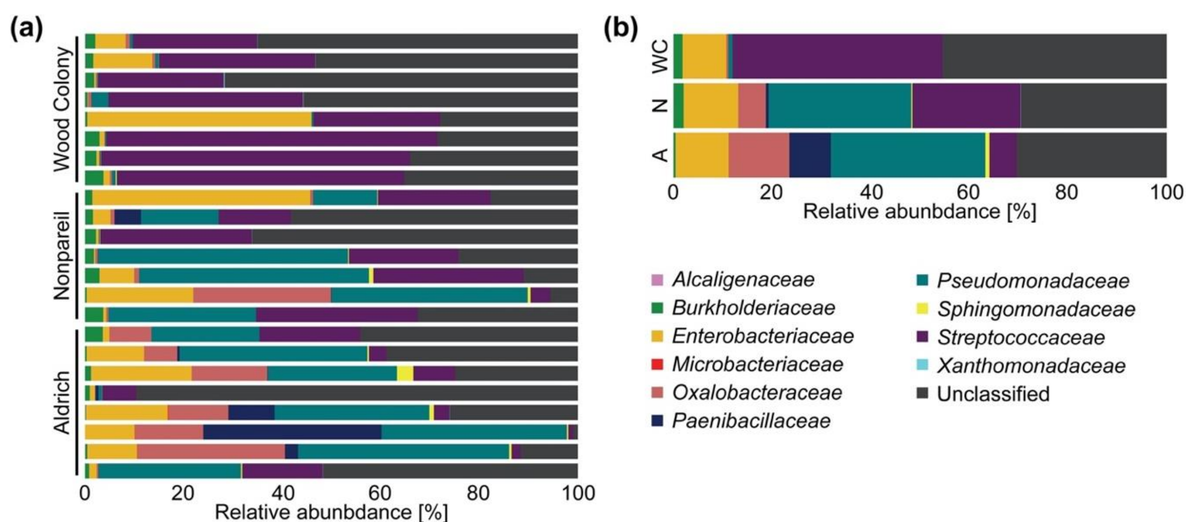

Figure 3-5. Relative bacterial family abundance of the three cultivars analyzed.
(a) Taxonomic composition of leaf endophytic bacteria of each individual almond tree tested. (b) Mean leaf bacterial endophyte composition of each cultivar (A: Aldrich, N: Nonpareil, WC: Wood Colony).

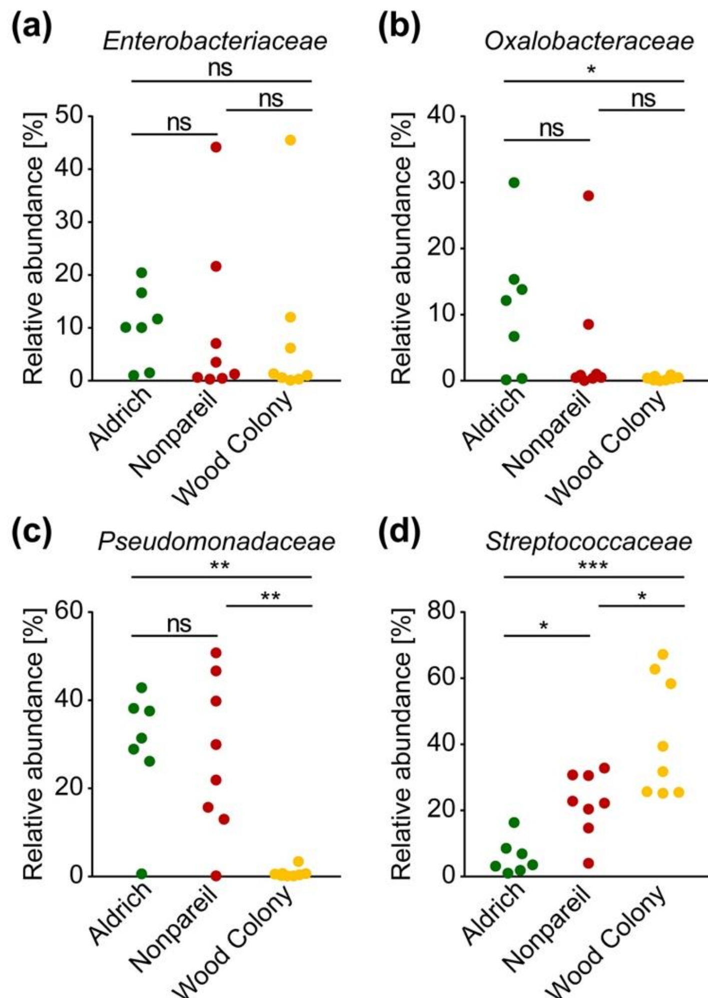

Figure 3-6. Comparison of the four most abundant bacterial families
Comparison of the four most abundant bacterial families among all samples. (One-way ANOVA, Tukey's multiple comparisons test; *, ** and *** denote a significant difference at α = 0.05, 0.01 and 0.001, respectively; ns denotes not significant at α = 0.05).

3.5 Discussion

Climatic factors, land management, soil type, and nutrient availability determine the bacterial communities in the environment that could potentially colonize plants (Hardoim et al., 2015). Bacteria from sources like bioaerosols, precipitation, and animals (such as pollinators and herbivores) are able to colonize aboveground plant tissues (Weintraub and Beanland, 2005; DeLeon-Rodriguez et al., 2013; Fröhlich-Nowoisky et al., 2016; Frank et al., 2017). Soil is considered the

most important source of below- and above-ground endophytes (Compant et al., 2010; Hardoim et al., 2015).

In soil, roots shape their surrounding microbiota by releasing exudates, comprised of sugars, proteins, and fatty acids, that are important sources of nutrients for soil microorganisms (de Weert et al., 2002; Bais et al., 2006; Badri and Vivanco, 2009). Bacteria can enter the root systems through lesions or emerging lateral roots (Chi et al., 2005; Compant et al., 2005). Once inside the root, bacteria are able to move throughout the plant via vascular systems (Compant et al., 2010; Hardoim et al., 2015). How bacterial endophytic leaf populations are assembled is not fully understood but, besides environmental factors, the host species is an important determinant (Redford et al., 2010; Kim et al., 2012; Kembel and Mueller, 2014; Kembel et al., 2014). Our results are especially surprising because all three cultivars not only shared a common environment but were also grafted to the same rootstock type (Nemaguard). Therefore, our study suggests that the shoot tissue genotype alone can shape the leaf endophytic community composition of almond trees. Our findings suggest that bacterial colonization itself, or the subsequent establishment inside the leaf, is dependent on the genetic differences among almond cultivars. This is in contrast to a recent study that compared bacterial endophytes of shoot xylem tissues in three apple cultivars (Liu et al., 2018). No statistically significant differences were observed among these cultivars. Species- (almond vs. apple) or tissue-specific (leaf vs. shoot xylem) may explain the differences between these two studies.

The exact genetic origin of the three almond cultivars used in our study is unknown. According to historical records, Nonpareil was introduced to California in the 19th century, likely from southern France (Kester and Gradziel, 1996; Bartolozzi et al., 1998; Gradziel, 2011). The genetic origins of Aldrich and Wood Colony are also unclear; both cultivars were found as chance seedlings in California in the second half of the 20th century (Aldrich, 1984; Blickenstaff, 1985). Aldrich has a bloom time that coincides with that of Nonpareil; hence, it is hypothesized to have originated from a cross between Nonpareil and Mission

(Aldrich, 1984). This hypothesis is supported by Simple Sequence Repeat marker analyses (Dangl et al., 2009). In contrast, Wood Colony blooms later than Nonpareil and Aldrich. Additionally, Wood Colony's shell and kernel are similar to those of the cultivars Carmel and Ne Plus Ultra (Blickenstaff, 1985). These phenotypic traits suggest that Wood Colony is phylogenetically less closely related to Nonpareil than Aldrich. The genetic dissimilarities between Wood Colony and the other two cultivars might explain the observed differences in bacterial community composition.

Our study is the first that compares leaf-derived bacterial endophytes among almond cultivars. To the best of our knowledge, there are only two other reported analyses of almond leaf surface bacteria. These studies describe 12 (Theofel et al., 2020) and 13 (McGarvey et al., 2019) predominant bacterial families, respectively, inhabiting almond leaf surfaces. Interestingly, our dataset shares only 3 out of 12 (*Enterobacteriaceae*, *Microbacteriaceae* and *Sphingomonadaceae*) and 5 out of 13 (*Enterobacteriaceae*, *Microbacteriaceae*, *Oxalobacteraceae, Pseudomonadaceae* and *Sphingomonadaceae*) of these bacterial families. These results indicate that the bacterial community composition inside and outside of almond leaves differ substantially.

We found that endophytic communities of Nonpareil and Aldrich were dominated by sequences representative of *Pseudomonadaceae*. This bacterial family contains several pathogenic strains causal for a plethora of bacterial plant diseases. For example, *Pseudomonas syringae* pv. *syringae* is responsible for bacterial canker in almonds (Palacio-Bielsa et al., 2017), and produces syringomycin, a phytotoxin that forms pores in cell membranes. This phytotoxin results in plant cell leakage and ultimately death, providing nutrients for this apoplastic pathogen (Bender et al., 1999; Höfte and De Vos, 2007). Bacterial canker is also known as "sour sap," because infected trees can produce a watery sap that runs down the trunk, resulting in brown, moist and sour smelling wood. Eventually, *Pseudomonas syringae* pv. *syringae* kills branches or the entire tree (Palacio-Bielsa et al., 2017). Additionally, *Pseudomonas amygdali* is the cause of

hyperplastic canker, which starts with canker formation on branches and trunks. The affected area can split longitudinally, leading to an open canker that can grow up to 20 cm. The infected trees show a reduction in growth, leading to almond yield losses (Psallidas, 1997; Palacio-Bielsa et al., 2017). Both *Pseudomonas* species are accountable for enormous yield losses not only in almonds, but in many diverse crops around the globe (Höfte and De Vos, 2007; Kennelly et al., 2007). Interestingly, in contrast to Aldrich and Nonpareil, *Pseudomonadaceae* accounted only for less than 1% of the endophytic bacteria composition of Wood Colony. Instead, *Streptococcaceae* was the predominant family in Wood Colony; this bacterial endophytic family exhibited a lower relative abundance in Nonpareil and Aldrich.

Streptococcaceae are not known to be causal for almond diseases. Currently, it is unclear if there are any antagonistic effects between *Streptococcaceae* and *Pseudomonadaceae* that could explain our observed patterns in relative abundances of bacterial endophytic in the almond cultivars. However, it has been shown that multiple commensal *Streptococcus* species have the ability to suppress growth of human pathogenic *Pseudomonas* strains through the production of hydrogen peroxide (Scoffield and Wu, 2015; Whiley et al., 2015). Furthermore, filtered supernatant of *S. sanguinis* culture exhibit a bactericidal effect on multiple *Pseudomonas* strains (Watanabe et al., 2009), and *S. parasanguinis* can utilize an exopolysaccharide to promote its own biofilm production while suppressing *P. aeruginosa* growth (Scoffield et al., 2017). Currently, it is unknown if similar antagonisms between these two bacterial families exist in plants as well. Isolation of different *Streptococcaceae* strains from almonds would allow the evaluation of whether or not these bacteria are able to inhibit growth of phytopathogenic microbes. Potential mechanisms by which *Streptococcaceae* strains could suppress potential phytopathogenic bacteria include competition for space and nutrients, induction of host resistance, and production of antibiotic components, as has been shown for other bacteria (Legein et al., 2020). Regardless of the mechanism, if *Streptococcaceae* strains do indeed

suppress phytopathogenic bacteria in almonds, application of these *Streptococcaceae* strains could serve as biocontrol agents to minimize the occurrence of *Pseudomonas*-derived and possibly other almond diseases. Additionally, it would be of great interest to find other *Prunus* cultivars with high relative abundance of *Streptococcaceae* strains and low relative abundance of *Pseudomonadaceae* species as leaf endophytes. The identification of the underlying genetic causes for these interactions would allow the inclusion of these traits into breeding strategies and contribute to a more sustainable agriculture.

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4 Characterization of *Erwinia gerundensis* A4, an almond-derived plant growth promoting endophyte

4.1 Abstract

The rapidly increasing global population and anthropogenic climate change have created intense pressure on agricultural systems to produce increasingly more food under steadily challenging environmental conditions. Simultaneously, industrial agriculture is negatively affecting natural and agricultural ecosystems due to intensive irrigation and fertilization to fully utilize the potential of high-yielding cultivars. Growth promoting microbes that increase stress tolerance and crop yield could be a useful tool for helping mitigate these problems. We investigated if commercially grown almonds might be a resource for plant colonizing bacteria with growth promotional traits that could be used to foster more productive and sustainable agricultural ecosystems. We isolated an endophytic bacterium from almond leaves that promotes growth of the model plant *Arabidopsis thaliana*. Genome sequencing revealed a novel *Erwinia gerundensis* strain (A4) that exhibits the ability to increase access to plant nutrients and to produce the stress mitigating polyamine spermidine. Because *Erwinia gerundensis* is able to colonize diverse plant species including cereals and fruit trees, A4 may have the potential to be applied to a wide variety of crop systems.

4.2 Introduction

Climate change and the growing world population create increasing pressure for agricultural systems to produce sufficient food (United Nations, 2019). To compensate for this demand, modern agriculture uses high-yielding cultivars and applies abundant irrigation water, fertilizers and pesticides (Martinho, 2020). Irrigation water is not only a limited resource, but its excessive use can also negatively impact yield and the environment. For example, intensive irrigation can lead to loss of fertile soils and increased soil salinity (Dale and Polasky, 2007). Additionally, these surface runoffs can contaminate surface water as well as groundwater (Dale and Polasky, 2007; Martinho, 2020).

Almonds are an example of a high demanding agricultural product (Zhang et al., 2019). They are important for diverse culinary cultures around the world and their consumption can improve cholesterol and blood sugar levels (Li et al., 2011; Ortiz et al., 2012; Choudhury et al., 2014; Berryman et al., 2015). These positive attributes among others have led to a high market demand that requires large fertilizer inputs and ample amounts of water usage to fully utilize the almond trees' yield potential (Zhang et al., 2019). For example, almond tree irrigation requirements are high, with estimates of twelve liters of water to produce a single almond kernel (Fulton et al., 2019). To mitigate the direct and indirect negative impacts of modern agriculture on natural and agroecosystems, more sustainable strategies are required. Utilizing bacteria could be a possible approach to counter the negative impacts of modern agriculture (Compant et al., 2010). Diverse bacteria living in association with plants are able to increase their host's stress tolerance and nutrient availability and, therefore, could reduce the need for irrigation and fertilizer inputs (Sturz et al., 2000; Lodewyckx et al., 2002; Ryu et al., 2004; Chen et al., 2010; Yandigeri et al., 2012; Timmusk et al., 2014; Soares et al., 2016; Verma et al., 2018).

Microbes are crucial for the bioavailability of phosphorus (P) for plants, because the production of organic acids leads to the release of recalcitrant P (Kafle et al., 2019). For example, bacterial glucose dehydrogenase is encoded by *gcd*

gene and uses glucose for the synthesis of gluconic acid (Goldstein, 1995; Liang et al., 2020). This enzyme requires the bacterial redox active cofactor pyrroloquinoline quinone (PQQ), which is produced by six Pqq proteins (Anthony, 2001; Shen et al., 2012). The presence of *gcd* and the *pqq* cluster indicates a bacterial strains ability to lower the pH of alkaline soils in order to increase availability of phosphorous and iron. Furthermore, bacteria are able to produce siderophores, low-molecular mass molecules with a high affinity for iron (Fe; Richardson et al., 1999). For example, enterobactin has an exceptionally high affinity for Fe^{3+} ($K_a = 10^{52}$), and is synthesized by several enzymes encoded by *ent* genes (Avdeef et al., 1978; Raymond et al., 2003; McRose et al., 2018). Several studies have shown that plants are able to access Fe by the uptake of microbial siderophores (Bar-Ness et al., 1992; Vansuyt et al., 2007; Jin et al., 2010; ShirleyMatt et al., 2011).

Plants synthesize low-molecular-mass linear polyamines, like spermidine, that are essential for plant growth (Kusano et al., 2008). Supplemental spermidine, provided either by overexpression of spermidine synthetase or exogenous application, enhances plant defense responses and increases tolerance to diverse abiotic stresses, like salinity and drought (Yoda et al., 2003; Kasukabe et al., 2004; Moschou et al., 2009). Overall, these findings suggest that increasing spermidine could improve plant tolerance to diverse environmental stresses. Bacteria are a potential source for providing supplemental spermidine to plants (Xie et al., 2014). They can synthesize spermidine from the two amino acids, methionine and arginine, catalyzed by enzymes encoded by the *metK* and *spe* genes (Tabor and Tabor, 1985; Shah and Swiatlo, 2008; Guerra et al., 2018).

To identify potential resources for a more sustainable agriculture, the primary goal of this study was to isolate and characterize a growth promoting bacterial endophyte from almond leaves that carries genes to improve nutrient availability and plant stress tolerance. We successfully isolated a growth promoting bacterium, and whole genome sequencing analysis identified this bacterium as novel strain of *Erwinia gerundensis*. This bacterial species has been found in diverse agricultural

ecosystems around the globe. This suggests that the bacterial strain described here has a potential use not only in almonds, but for a wide variety of other crops.

4.3 Methods

4.3.1 Sample collection and leaf tissue sterilization

Leaves of visually healthy almond trees growing in an orchard located in Modesto, California (37°42'21.8"N, 120°56'55.1"W) were collected aseptically in July 2019. Leaves were placed in sterile bags and immediately stored at 4 °C in a portable cooler, and then transported to the laboratory at the University of California, Merced. Within 3 hours after collection, leaves were surface sterilized using 8.25% sodium hypochlorite. Removal of epiphytic microbes was confirmed using scanning electron microscopy (SEM). Surface sterilization and SEM procedures were previously described for cottonwood leaves (Saldierna Guzmán et al., 2020).

4.3.2 Enrichment and isolation of endophytic bacterial strains

Immediately after removing epiphytic microbes, 20 g of fresh and surface sterilized leaves were blended for 3 min to enrich for endophytic bacterial cells (Ikeda et al., 2009). Due to minor accumulation of cell debris, we excluded the overlay on nycodenz step. The enriched endophytic bacterial cells were resuspended using 1x phosphate buffered saline (PBS). Ten µl of this suspension was plated on Lysogeny Broth (LB) agar media and Norris Glucose Nitrogen Free Media (HIMEDIA, Mumbai, Maharashtra, India), and then incubated 5-7 days at 28 ^{o}C. Subsequently, the obtained colonies were repeatedly streaked and incubated on agar-media to obtain pure isolates. We isolated a total of 100 bacterial strains from surface sterilized almond leaves. Of these bacteria, 25 strains were randomly selected and tested for their plant growth promoting abilities. Only one of these 25 strains had a positive effect on plant growth.

In order to identify the isolates, 16S rRNA gene amplification of each sample was conducted using universal primers 27f and 1492r (Lane, 1991). The 50 µl PCR reaction contained 1X complete reaction buffer, 200 µM dNTPs, 0.5 µM forward primer, 0.5 µM reverse primer, 10 ng of template DNA, and 1.25 units DFS-Taq DNA polymerase (BIORON GmbH, Römerberg, Rheinland-Pfalz, Germany). Cycling conditions were 95 °C for 2 min, followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min, with a final extension of 72 °C for 10 min. All samples were purified from 1.5% agarose gels using the Zymoclean Gel DNA Recovery Kit (Zymo research, Irvine, CA, USA) following the manufacturer's recommended procedure. Subsequently, amplicons were sent for Sanger sequencing at Eton Bioscience (San Diego, CA, USA).

4.3.3 Competent cells and transformation of endophytic bacterial strain

To generate competent cells of the endophytic strain A4, cells were grown overnight at 28 ºC until they reached an optical density (OD_{600}) of 0.5. They were cooled in an ice bath and washed with sterile, cold water four times after centrifugation steps at 4000 g. Afterwards, cells were resuspended in 10% glycerol, aliquoted and snap frozen in liquid nitrogen. Electroporation was used for transformation of A4 with the pRU1156 plasmid (Karunakaran et al., 2005).

4.3.4 Western blotting

Transformed and untransformed A4 strains were grown on LB liquid media overnight at 28 °C. Bacterial suspensions were normalized by measuring OD_{600}, and equal amounts of cells were boiled in sample loading buffer (Sigma-Aldrich, Darmstadt, Germany) and separated in SDS-PAGEs. For immunoblotting, anti-GFP (1:5000, Roche, 11814460001, Darmstadt, Germany) was used to detect free GFP (Bürger et al., 2017).

4.3.5 Plant inoculation

Arabidopsis thaliana (ecotype Columbia-0) seeds were surface sterilized by hydrochloric fumigation for 3 h and stratified for 3-5 days at 4 ºC (Lindsey et al., 2017). Subsequently, the sterile seeds were grown *in vitro* on half-strength Linsmaier and Skoog (LS) medium at pH 5.7 with 7% plant agar. Seedlings were grown on vertically oriented plates in a growth chamber (Percival, Iowa, USA) with continuous white light (100 µmol m^{-2} s^{-1}) at 21 ºC (Qiu et al., 2019). Prior to inoculation, the endophytic strain was grown for 24 h in LB media with 50 µg tetracycline ml^{-1} and 100 µg ampicillin ml^{-1} at 28 ºC. The bacterial cells were centrifuged at 3500 g, resuspended in infiltration buffer (10 mM $MgSO_4$, 10 mM MES-KOH pH 5.5) and adjusted to an OD_{600} of 0.1 in the same buffer. Subsequently, the roots of 7-day old seedlings were inoculated with bacterial inoculum, being careful not to contaminate shoot tissues, following the protocol for *S. enterica* and *E. coli* O157:H7 (Cooley et al., 2003). Infiltration buffer without bacteria served as the negative control (mock treatment).

4.3.6 Growth promotion assay

The effect of bacteria on *Arabidopsis* growth was evaluated two weeks post-inoculation. Three independent experiments were performed with 38 to 41 seedlings per treatment in each experiment. Fresh mass of seedlings was measured and compared to mock (infiltration buffer) treated plants, which served as negative control. Statistical analysis was performed by one-way ANOVA with Tukey's test using GraphPad Prism 7 (GraphPad Software, La Jolla California, USA). A difference was considered statistically significant using an a priori determined α level of 0.05.

4.3.7 GUS and GFP

In order to test colonization of the endophytic strain A4, plant tissues were histochemically stained with X-Gluc (GUS) seven days after inoculation of *A. thaliana* seedlings, and observed with a stereomicroscope (Leica MZ16, Leica,

Germany; Willige et al., 2011). Moreover, seedlings were screened for green fluorescent protein (GFP) expression using a Zeiss LSM 710 fluorescent microscope (Carl Zeiss, Germany).

4.3.8 Genome sequencing

The bacterial strain A4 was sent to Novogene Biotech (Beijing, China) for DNA extraction, library preparation, and whole genome sequencing and assembly using PacBio Single Molecule, Real-Time (SMRT) Sequencing (Eid et al., 2009). Falcon software (Chin et. al., 2016) was used for genome assembly, and BUSCO for the assessment of the genome assembly, gene set, and transcriptome completeness (Simão et al., 2015). Prokka software was used for genome annotation (Seemann, 2014), and functional annotation was done by aligning the sequence with sequences previously deposited in diverse protein databases, including: the National Center for Biotechnology Information (NCBI) non-redundant protein (Nr) database, UniProt/Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Cluster of Orthologous Groups of proteins (COG).

4.3.9 Bioinformatics

The phylogenetic tree was constructed with SpeciesTreeBuilder v 0.1.0 from the Kbase platform (Arkin et al., 2018). The Artemis Comparison Tool (ACT; Carver et al., 2005) was used to compare our bacterium genome with an already sequenced genome. To display circular comparisons between both genomes and plasmids, the Blast Ring Image Generator (BRIGS) was used (Alikhan et al., 2011). Finally, a nucleotide identity analysis between A4 and a publicly available, closely related bacterial genome was performed with the ANI calculator from EZbiocloud (Yoon et al., 2017).

4.3.10 Phosphate solubilization

A4 was tested for its ability to solubilize phosphate using NBRIP medium (Nautiyal, 1999). Prior to inoculation, A4 cells were grown at 28 ºC until they

reached an OD_{600} of 0.5. An aliquot of 10 µl was spotted on plates containing NBRIPM medium and incubated at 28 ºC for seven days.

4.3.11 Siderophore

Siderophore production was determined by chrome azurol S (CAS) agar plates. The medium was prepared according to the method described by Schwyn and Neilands (1987). A4 was grown overnight in LB liquid medium at 28 °C. A4 suspension was normalized by adjusting to an OD_{600} of 0.5 with LB medium. Subsequently, 10 µl of the suspension was spotted on CAS agar plates and incubated seven days at 28 ºC.

4.4 Results

4.4.1 Effect of A4 endophyte on plant growth

Sanger sequencing of the 16S rRNA gene of the bacterial strain isolated from surface sterilized almond leaves revealed its phylogenetic relationship to the genera *Pantoea* and *Erwinia*. We named this bacterial strain A4. Three independent experiments revealed that endophyte-treated plants had about 30% higher fresh mass than control plants ($p \leq 0.001$; Figure 4-1a, b). Additionally, the A4 treatment resulted in increased root hair length and abundance (Figure 4-c, d). These results suggest that inoculation with an almond-derived endophyte could promote growth of *Arabidopsis* seedlings.

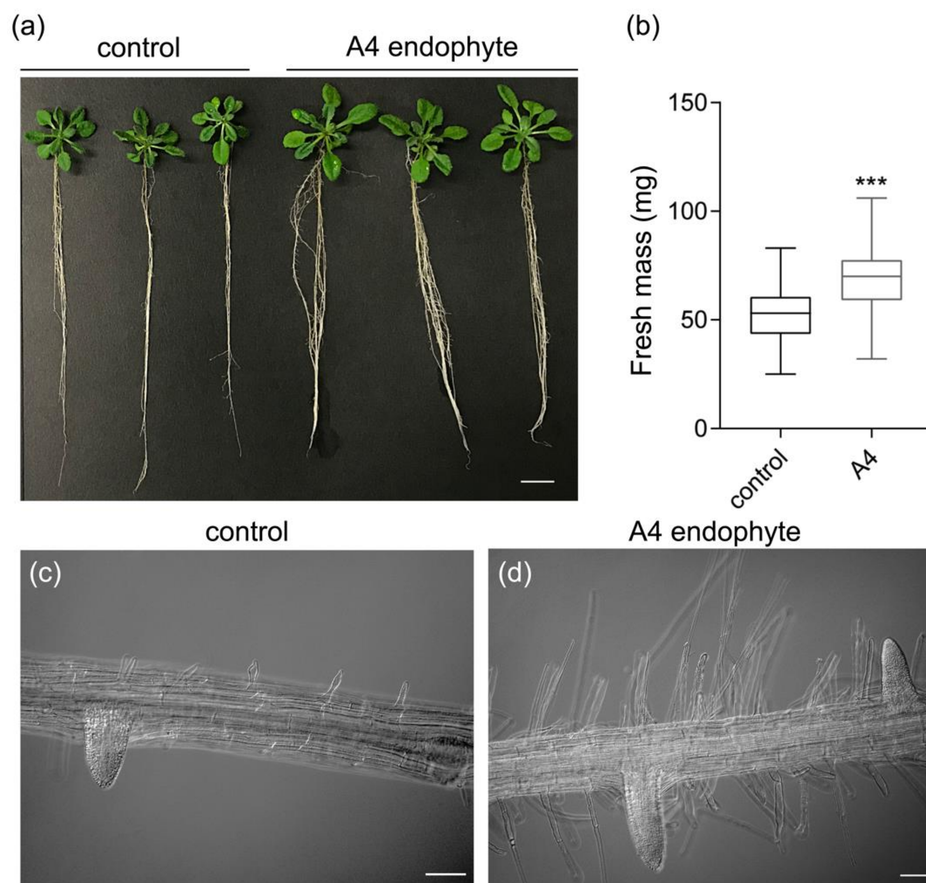

Figure 4-1. Effect of endophytic strain A4 on *Arabidopsis thaliana*
(a) Photo taken after 2 weeks post-inoculation in comparison to the control. Scale bar = 1 cm. (b) Fresh weight of seedlings compared to the control (One-way ANOVA, Tukey's multiple comparisons test, *** $p \leq 0.001$), n = 41. (c) and (d) Differences in the root after 2 weeks of inoculation with A4 in contrast to the control. Scale bar = 0.2 mm. Control for all experiments: mock (infiltration buffer) treated plants.

4.4.2 Transformation of A4-strain

A4 was isolated from surface sterilized almond leaves, which indicated that the strain was an endophyte. To test plant colonization by A4, we transformed A4 to express the marker genes GFP and gusA in order to track the colonization of A4 within internal plant tissues. Successful transformation of A4 was confirmed by immunoblotting to visualize expression of gfp encoded in the transformed plasmid (Figure 4-2a).

Arabidopsis roots were inoculated with endophytic bacteria A4-labeled with green fluorescent protein (GFP) to determine whether intracellular colonization was occurring by live-cell imaging. Confocal observation revealed GFP-expressing

A4 colonizing internal root tissues seven days after inoculation. Many of these bacterial cells were observed within the root vasculature (Figure 4-2b). Furthermore, analyses 14 days post-inoculation showed that A4 had colonized shoot tissues such as leaves and flowers, as visualized by GUS-staining (Figure 4-2c, d). These observations indicated that the root-inoculated bacteria were able to spread inside the plant and colonize above ground tissues. The mock-inoculated plants did not show any blue staining or fluorescent bacteria.

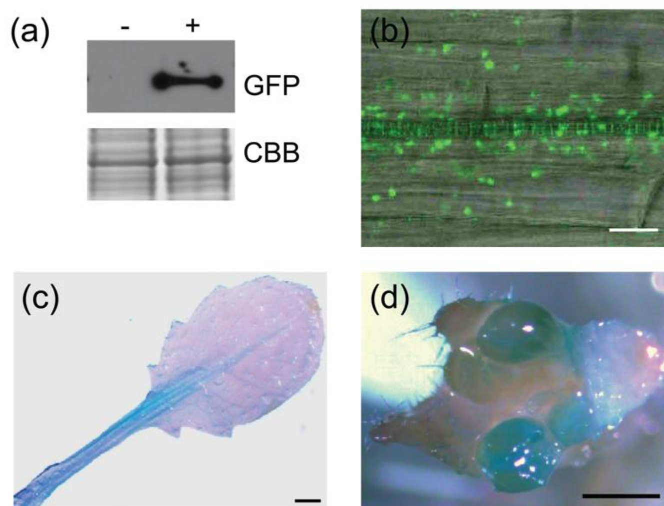

Figure 4-2. Colonization of the endophytic strain A4
Western blot analysis of A4 transformant expressing green fluorescent protein (GFP). Coomassie stain (CBB) shows equivalent protein loading. An antibody against GFP was used for visualization and non-transformant A4 strain as a negative control. Seven days after inoculation of *Arabidopsis thaliana* seedlings, (b) GFP-expressing A4 inside the primary root. Scale bar = 20 μm. (C) Histochemical GUS activity of A4 strain in (c) leaf tissue and (d) inflorescence. Scale bar = 1 mm.

4.4.3 Genomic features of A4

The whole-genome sequence analysis of A4 was conducted in order to obtain reliable taxonomic classification and identify genes or pathways that could potentially contribute to the plant growth promoting effects. The A4 genome consisted of a single circular chromosome of 3,858,052 bp, with an average GC content of 55%. The A4 strain had one plasmid we named pA401, with a size of approximately 576,382 bp, and also had an average GC content of 55% like the chromosome. The chromosome contained 3,552 genes, including genes for 78

tRNAs, for 22 rRNAs, and 3,451 protein-coding sequences (CDS). Among these CDSs, 2,929 genes were classified into clusters of orthologous groups (COG) families comprised of 23 categories. KEGG pathway annotation resulted in the functional annotation of 3,414 genes (96%). Of these annotated genes, most were grouped into the two categories “metabolism” and “environmental information processing” (Table 1). The A4 plasmid pA401 had 521 protein-coding sequences and 1 contig.

Table 1. Genome features of A4

COG pathway annotation		**KEGG function classification**	
Function	**Genes**	**Function**	**Genes**
RNA processing and modification	1	**Genetic information**	
Energy production and conversion	160	Translation	81
Cell cycle control and division, chromosome partitioning	44	Transcription	4
Amino acid transport and metabolism	341	Replication and repair	54
Nucleotide transport and metabolism	92	Folding, sorting and degradation	51
Carbohydrate transport and metabolism	307	**Cellular processes**	
Coenzyme transport and metabolism	168	Transport and catabolism	8
Lipid transport and metabolism	113	Cellular community	151
Translation, ribosomal structure and biogenesis	239	Cell mobility	90
Transcription	247	Cell growth and death	21
Replication, recombination and repair	127	**Metabolism**	
Cell wall/membrane/envelope biogenesis	223	Xenobiotics biodegradation and metabolism	26
Cell motility	110	Nucleotide metabolism	115
Posttranslational modification, protein turnover, chaperones	126	Metabolism of terpenoids and polyketides	32
Inorganic ion transport and metabolism	208	Metabolism of other amino acids	61
Secondary metabolites biosynthesis, transport and catabolism	62	Metabolism of other cofactors and vitamins	157
General function prediction	253	Lipid metabolism	60
Signal transduction mechanisms	186	Glycan biosynthesis and metabolism	48
Intracellular trafficking, secretion and vesicular transport	63	Energy metabolism	124
Defense mechanisms	63	Carbohydrate metabolism	233
Extracellular structures	18	Biosynthesis of other secondary metabolites	32
Mobile: prophages, transposons	15	Amio acid metabolism	174
Unknown	183	**Environmental information processing**	
		Signal transduction	123
		Membrane transport	248

4.4.4 Taxonomic affiliation of strain A4

We used Tree builder to search for closely related genomes and to assess the phylogenetic relationship between A4 and publicly available genomes (Figure 4-3). This analysis revealed that A4 does not belong to the genus *Pantoea*. A4 and the previously sequenced bacterium *Erwinia gerundensis* EM595 (Rezzonico et al., 2016) were both strains of the same bacterial species. Therefore, we compared chromosomes and plasmids of A4 and EM595 (Figure 4-4). While the chromosomes of both strains share about 90% of their genes (3175), A4 had 224 unique genes. Furthermore, EM595 carried two plasmids (pEM01 and pEM02),

and pEM01 shared the greatest overlap with pA401 (71%, 463 genes); 20 of the common genes were also present on pEM02. Additionally, the A4 plasmid had 48 unique genes that were not present in both plasmids of the previously sequenced *Erwinia* strain EM595. Taken together, these results suggest A4 represents a novel *Erwinia gerundensis* strain.

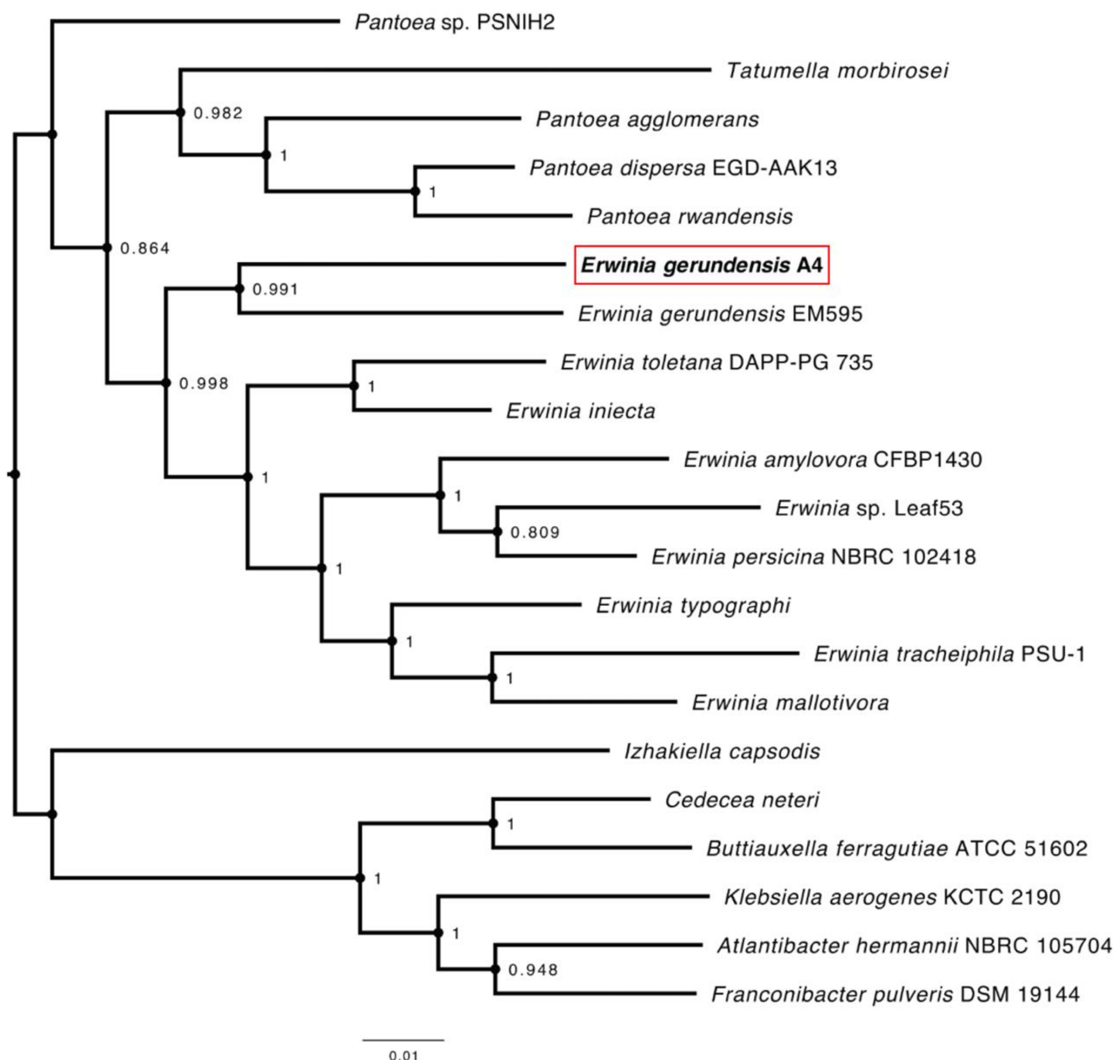

Figure 4-3. Phylogenetic tree highlighting the position of Erwinia gerundensis A4 in comparison to publicly available genomes. Node labels represent confidence levels based on 1000 bootstrap replicates.

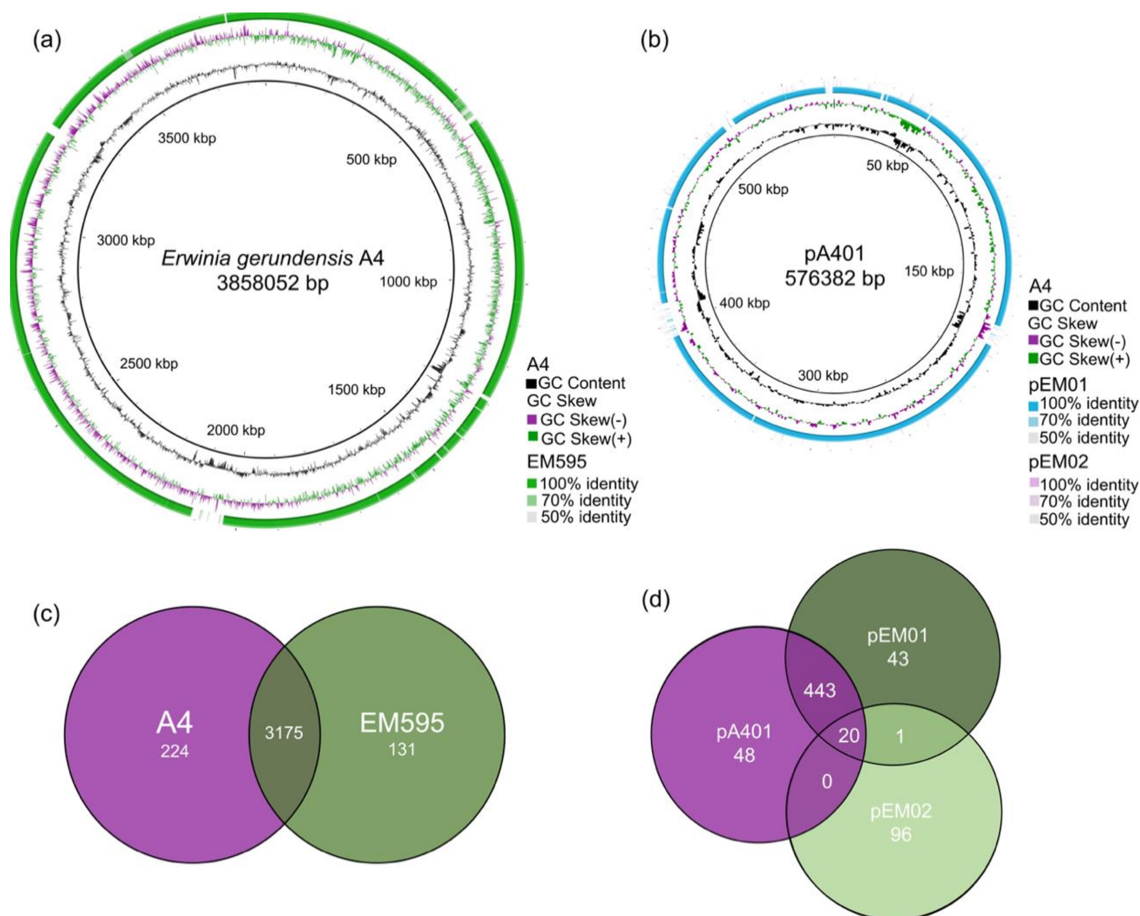

Figure 4-4. Comparison between *Erwinia gerundensis* A4 and EM595
(a) and (b), Circular comparisons between both genomes and plasmids. The inner black rings depict the coordinates in scale and total size of chromosome (a) and plasmid (b) of A4. Black histograms represent GC content, while green-purple histograms show GC deviations. Orthologous sequences are displayed with the percentage of similarity. (c) and (d), Venn diagrams of chromosomes and plasmids of both strains.

4.4.5 Plant Growth Promoting Traits in A4

In order to identify genes responsible for growth promotion, we searched the genome and plasmid of A4 for genes involved in nitrogen fixation, and organic acid, siderophore, and spermidine synthesis. Even though A4 was isolated on bacterial medium that is denoted as nitrogen free, we could not detect any genes encoding for nitrogenase subunits. However, we identified a *pqq* gene cluster (*pqqABCDEF*) as well as for a *gcd* gene, indicating that A4 carries all necessary enzymatic components to solubilize phosphorus by organic acid synthesis. Moreover, we found *entABCDEF* and two *entS* genes in A4, suggesting that this

strain is able to produce and export the siderophore enterobactin. Furthermore, the strain carries all necessary genes (i.e., *metK* and *speABDE*) to produce the polyamine spermidine. In addition, we identified several genes involved in spermidine and putrescine transport (i.e., *potA*, *potB*, *potD*, *potG*, *potH* and *potI*; Figure 4-5).

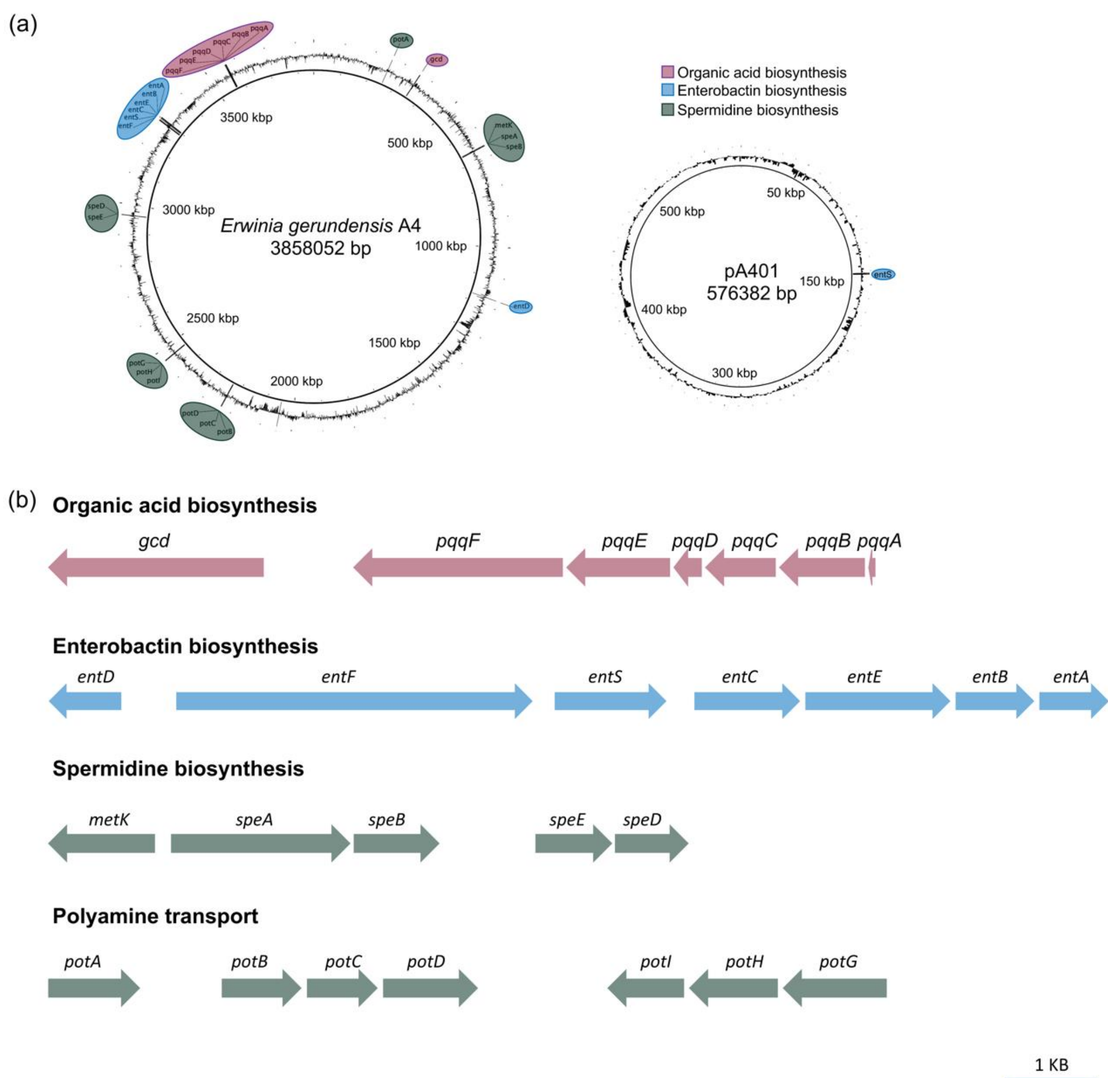

Figure 4-5. Genes encoding for plant promoting traits in *Erwinia gerundensis* A4
(a) Location and (b) cluster of genes encoding production and transport of spermidine, the siderophore enterobactin, and the phosphate solubilizing gluconic

4.4.6 Nutrient acquisition by A4 strain

Because the genome of A4 encodes for proteins that are involved in the production of organic acids and siderophores, we tested if A4 is able to release insoluble phosphate and synthesize iron chelators. A4 was able to produce a clear zone around the colony suggesting phosphate solubilization activity by the production of organic acids. Additionally, production and secretion of siderophores was visualized by a color change of Chrome Azurol S (CAS). In association with ferric ions, CAS appears blue, while the removal of the ferric ions by siderophores changes the color of the media from blue to yellow. Consistent with the presence of genes encoding for siderophore synthesizing enzymes, A4 was able to cause the color change of the CAS media.

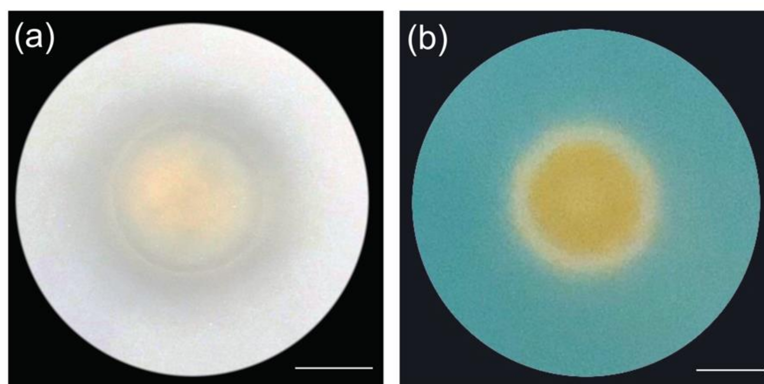

Figure 4-6. Nutrient acquisition abilities by A4
(a) Phosphate solubilization by A4. Clear zone surrounding the bacterial colony represents solubilization of $Ca_3(PO_4)_2$. (b) Siderophore production in CAS medium. The color change from blue to yellow caused by siderophore production can be observed surrounding the bacterial colony. Scale bars = 10 mm.

4.5 Discussion

Bacteria living in association with plants can either be harmful, neutral, or beneficial. Once a beneficial bacterium has been established on or in a plant, it can influence the host's physiology via a variety of mechanisms. For example, bacteria can produce growth promoting metabolites, easing the effects of biotic and abiotic stresses and providing nutrients (Bulgarelli et al., 2013). Here, we

described the isolation, characterization and sequencing of the growth promoting *E. gerundensis* strain A4. This strain was isolated from inner leaf tissues from almonds growing in a commercial orchard. So far, only one other *E. gerundensis* strain has been sequenced, which was isolated from leaf surfaces of a pear tree (Rezzonico et al., 2016). Our sequence analysis identified A4 as a novel *E. gerundensis* strain. Furthermore, whole genome sequencing revealed that A4 exhibits the ability to produce polyamines, gluconic acid, and enterobactin.

Polyamines, like spermidine, are organic compounds produced by prokaryotes and eukaryotes that are essential for their growth and development (Kusano et al., 2008). In plants, they play a role in a variety of process like embryogenesis, root growth, flowering, fruit maturation, and retardation of senescence (Evans and Malmberg, 1989; Pandey et al., 2000). For example, in *Arabidopsis thaliana*, loss of spermidine synthase activity leads to embryo lethality (Imai et al., 2004). Other than participating in plant growth, polyamines have a protective role against several environmental stresses. Kasukabe et al. (2004) showed that overexpression of plant-derived spermidine synthetase confers tolerance to freezing, salinity, drought, and osmotic stresses in *Arabidopsis thaliana*. Additionally, several studies have shown that polyamines are involved in defense responses against pathogens. The upregulated expression of polyamine synthesis genes in response to pathogenic attacks has been observed in several plant species (Fu et al., 2011; Moselhy et al., 2016). Furthermore, overexpression of spermidine synthetase or exogenous application of spermidine enhances plant defense responses to viruses and bacteria (Yoda et al., 2003; Moschou et al., 2009). Altogether, these studies indicate that increasing polyamine levels minimizes the detrimental effects caused by biotic and abiotic stresses. A4 could provide spermidine and ease the damaging effects of various environmental stresses, because it not only carries the genes for spermidine synthesis, but also for the export of the polyamines spermidine and putrescine.

Besides spermidine production, we identified several traits that may support nutrient acquisition of plants. For example, at a morphological level, A4 increases

root growth and the elongation and density of root hairs in *Arabidopsis*. These plant growth responses increase root surface area that can enhance the plant's access to water and the uptake of nutrients (Sukumar et al., 2013). Furthermore, A4's genome encodes for two enzymatic pathways involved in nutrient acquisition. Microbes developed diverse metabolic capacities to improve the bioaccessibility of recalcitrant P by the production of different organic acids (Richardson and Simpson, 2011). Bacterial PQQ-dependent glucose dehydrogenase, encoded by the *gcd* gene, catalyzes the oxidation of glucose to gluconic acid (Goldstein, 1995; Liang et al., 2020). Glucose dehydrogenase is characterized as a key enzyme necessary for phosphate solubilization in microbes. In fact, detection of the *gcd* gene in soil samples was found to be a major determinant of bioavailable P (Liang et al., 2020). Our functional analysis of A4 identifies genes encoding for the entire enzymatic pathway for synthesis of the redox cofactor PQQ and for gluconic acid production. We found that A4 is able to solubilize $Ca_3(PO_4)_2$, suggesting that gluconic acid is excreted by A4 to lower the pH of alkaline environments. In contrast, low soil pH leads to P fixation by iron. In acidic soils, bacteria can increase the plant's phosphate accessibility by producing siderophores that form complexes with ferric iron (Kafle et al., 2019). The genome and plasmid of A4 carry all necessary genetic information to produce and export the siderophore enterobactin. Our *in vitro* assay suggested that A4's *ent* genes are all functional. Several studies have demonstrated that plants are able to access Fe by the uptake of microbial siderophores (Bar-Ness et al., 1992; Vansuyt et al., 2007; Jin et al., 2010; ShirleyMatt et al., 2011). This indicates that siderophores produced by growth promoting bacteria have a dual function for the plants' nutrient availability in acidic soils by providing phosphate as well as iron. Therefore, A4 could exhibit plant growth promotion traits not only as a leaf endophyte where it could provide spermidine to foster stress tolerance, but also as a member of plant rhizospheres to ease access to nutrients. Furthermore, we observed floral colonization by A4. This opens the possibility that A4 might colonize reproductive tissues, and may suggest that A4 might be vertically transmitted from one generation to the next.

We isolated *E. gerundensis* strain A4 from inner tissues of almond leaves. In addition, other *E. gerundensis* strains were found in diverse agroecosystems around the globe. Two strains were isolated from leaf surfaces of pome fruit trees in Spain, and two other strains were isolated from wheat roots in Australia (Rezzonico et al., 2009, 2016). Taken together, these results suggest that *E. gerundensis* can colonize different tissues of various plant species, including both monocots and dicots, in diverse agricultural environments. This promiscuous colonization behavior and the growth promotion traits of *E. gerundensis* A4 suggest that this strain might have the potential to improve production not only of almonds, but also of a variety of other crop species around the globe.

4.6 References

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5 Conclusion

This dissertation applied diverse approaches to increase our knowledge about endophytic bacteria. First, I established sterilization methods for conifer and broad-leaved trees. This study highlights the importance of choosing the suitable sterilization protocol to prevent erroneous interpretation of host-endophyte interactions. Additionally, it demonstrates that electron microscopy is an appropriate tool for evaluating the effectiveness of surface sterilization methods. In contrast, the often-applied sterilization tests (media imprinting and PCR) were found to be unreliable, indicating the necessity to not use them as unequivocal measures of surface sterility in future studies.

Establishing these protocols was the prerequisite for analyzing almond endophytes by culture-dependent and -independent approaches. Using a commercial almond orchard to assess the bacterial leaf endophyte composition exhibited a very unique experimental set up. The trees grew in the same environment and were exposed to the same anthropogenic influence. Furthermore, they shared the same root genotype. To the best of my knowledge, this is the very first study that analyzed endophytic bacteria community compositions in different almond cultivars. My analysis suggests an antagonistic effect between *Pseudomonadaceae* and *Streptococcaceae*. In animal systems, multiple commensal *Streptococcaceae* species are able to suppress growth of pathogenic *Pseudomonadaceae* strains. These findings open up several future research directions. First, using the inhere established protocols will allow to isolate *Streptococcaceae* and *Pseudomonadaceae* strains from almond leaves

and test antagonistic interactions like competition for space and production of antibiotic components *in vitro*. Furthermore, application of endophytic *Streptococcaceae* strains in almonds could serve as biocontrol agents to suppress outbreaks of phytopathogenic bacteria. Finally, identifying the genetic causes in almonds for the acquisition of endophytic *Streptococcaceae* strains might allow the inclusion of these traits into almond breeding strategies.

Using a culture-dependent bacterial isolation method, I was able to obtain a growth-promoting leaf endophyte from almond leaves. Future studies will be necessary to establish agricultural strategies to efficiently utilize the positive effects caused by *E. gerundensis* A4. First, potential negative impacts of A4 on ecosystems or human health have to be evaluated. Second, it has to be assessed, if successful colonization by A4 is not only occurring in the model plant *Arabidopsis thaliana*, but also in crops. Third, studies need to evaluate if A4 can colonize the crop without being outcompeted by the plant's existing microbiome. Furthermore, appropriate bacterial inoculation methods for crops need to be developed that can be applied at an operational scale. Bacteria might need to be applied to the soil or on foliage. In addition to A4, other *E. gerundensis* strains have been isolated from root and leaf tissue from diverse crops around the globe. This not only highlights A4's potential to promote growth in various crop species, but also indicates that both inoculation strategies might be feasible. This is supported by my research showing that colonization of *Arabidopsis thaliana* by A4, demonstrating A4's movement from the rhizosphere to above ground tissues. Taken together, both approaches, culture-dependent and -independent, provide a foundation to develop strategies for a more sustainable agriculture utilizing endophytic bacteria.