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Isolation and Identification of Potential Bioinoculants based on Phosphate Solubilizing and Plant Growth Promoting Benefits

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ISOLATION AND IDENTIFICATION OF POTENTIAL BIOINOCULANTS BASED ON PHOSPHATE SOLUBILIZING AND PLANT GROWTH PROMOTING BENEFITS

BY

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This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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I would like to dedicate this to my husband who encouraged me and made me laugh every day of the last two years and helping keep Elam alive during this crazy time of life.

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ABSTRACT

ISOLATION AND IDENTIFICATION OF POTENTIAL BIOINOCULANTS BASED ON PHOSPHATE SOLUBILIZING AND PLANT GROWTH PROMOTING BENEFITS

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Conservative models have shown that as populations rise, food production needs to double by 2050. Population increase and the green revolution have caused fertilizer inputs to increase since the 1960's, increasing environmental issues and production costs. These intensive practices have led to degraded arable land and there has been an increase in urbanization meaning we need to make best use of the farming land that is available and ensure it is sustainable for future food production. Due to this, there has been a higher demand for research on more environmentally and economically friendly approaches to food production. Plant growth promoting bacteria have been a promising approach to more sustainable farming and have been shown to increase plant growth through characteristics such as; nitrogen fixation, plant hormone production, fungal suppression, and phosphate solubilization. It is estimated that only 0.1% of the phosphorus in the soil is in a soluble form, and approximately 80% of the phosphorus fertilizer applied gets bound in the soil and is not available to plants. Phosphate solubilizing bacteria can produce organic acids and phosphatase enzymes to solubilize soil phosphorus. Phosphate biofertilizers are one of the largest growing portions of the bioinoculant industry. In our research we have isolated and identified several potential plant growths promoting and phosphate solubilizing bacteria. In addition, we identified

some of the specific mechanisms used to increase plant growth, as well as the mechanisms to solubilize inorganic phosphorus.

Four novel isolates of the Oxalobacteraceae family were identified and characterized. Based on their phenotypic, morphological, and genomic analysis, three of the isolates are novel at the species level and one at the genus level. These isolates were cultured from agriculture and garden soils and have several genes that are considered plant growth promoting such as; nitrate reductase, urease, phosphatase, biotin production, decomposition on hydrogen peroxide, and biofilm biosynthesis. The four novel isolates are; *Pseudoherbaspirillum sperare* OM1, *Massilia arenosa* MC02, *Massilia hortus* ONC3, and *Duganella callidus* DN04.

Seventy bacteria were cultured from corn tissue, rhizosphere, and loose root soil and were screened for phosphate solubilizing abilities. Of the eight isolates tested, three bacteria were able to solubilize the highest level of phosphate; *Enterobacter cloacae* (Tr3R3), *Raoultella ornithinolytica* (M2R1), and *Kosakonia* sp. (Tc3So2). Of these three isolates, Tr3R3 and Tc3So2 had the highest soybean root and shoot biomass, root architecture, and plant phosphorus concentrations. Organic acid production was measured through HPLC and the two isolates that produced the greatest amount of soluble P, also produced the greatest amount of succinic acid. Additionally, the two isolated that did not solubilize any P, similarly did not produce any succinic acid, concluding that the succinic acid was the mechanism used to solubilize the phosphate. Through these tests as well as additional plant growth promoting tests such as nitrogen

fixation, indole acetic acid biosynthesis, and fungal suppression, we found that Tr3R3 and Tc3So2 are promising bioinoculants.

CHAPTER 1

INTRODUCTION

The world population is increasing and with that comes an increasing demand for food. By the year 2050, we will need to double our food production from the 2005 levels (Hunter, 2017). However, an agricultural expansion with the use of intensive practices will have compounding negative environmental impacts. This intensification of agriculture would increase the use of chemicals, and land clearing which ultimately increases greenhouse gas emissions and deforestation (Hunter, 2017). Ultimately, eco-friendly and economical solutions will need to be implemented to sustain the food production growth the world will need.

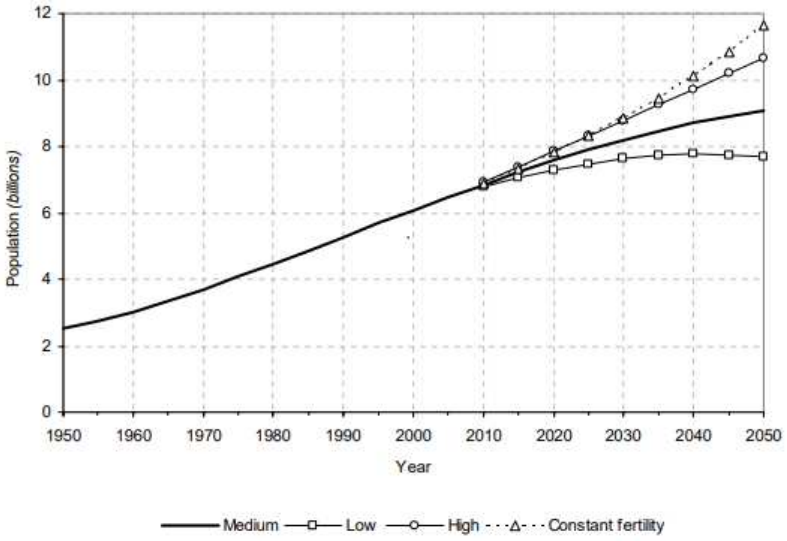
Bioinoculants are environmentally friendly alternatives to conventional farming. Due to the improvements in sequencing, we can culture and characterize novel isolates for bioinoculant commercialization. There are numerous benefits these bacteria bring to the plant, such as an increase in nutrient mineralization, indole-3-acetic acid production, nitrogen fixation, fungal suppression, siderophore production, ACC deaminase activity, and many more. These isolates have the potential to increase crop yield in a more sustainable way.

Due to phosphorus (P) adsorption to soil particles and binding to cations such as Fe, Al, and Ca the majority of P in the soil is unavailable to the plant (Richardson, Barea, McNeill, & Prigent-Combaret, 2009). Although phosphorus might be at adequate levels in the soil, approximately less than 0.1 % is in a form that is able to be assimilated by the plant (Zou, Binkley, & Doxtader, 1992).

Phosphate solubilizing bacteria (PSB) have been examined as an innovative approach to supply crops with adequate phosphorus (Goldstein, 1986). These microbes live on, in, and around the plant and can help to solubilize phosphorus into the plant available form orthophosphate, plant-useable form (Katznelson, Peterson, & Rovatt, 1962). The once, hard to dissolve P complexes, are able to be solubilized by phosphate solubilizing bacteria (PSB) by secreting organic acids and/or exo-enzymes (Richardson et al., 2009). These native PSB have been found to increase P-uptake, as well as significantly increase crop productivity (Richardson, Hadobas, Hayes, O'Hara, & Simpson, 2001).

WORLD FOOD DEMAND

From 1950 to 2000 the earth's population approximately doubled, from 2.5 to 6.1 billion, respectively. From 2000 to 2050 the population is estimated to increase from 6.1 billion to 9.1 billion (Figure 1) (UN, 2005). Another factor feeding into the population increase is life expectancy. With the increases in life span, it is estimated by the United Nations Department of Economic and Social Affairs, that from 2000 to 2050 the number of people 60 years of age and older will triple. This demographic shift will grow from 806 million to a remarkable 2 billion people (UN, 2001).



Source: Population Division of the Department of Economic and Social Affairs of the United Nations Secretariat (2005). *World Population Prospects: The 2004 Revision. Highlights*. New York: United Nations.

Figure 1 Population of the world, 1950-2050, by projection variants (UN, 2005).

With an increase in population comes an increase in food demand. The Food and Agriculture Organization of the United Nations, stated that in 2012, the total global agriculture food production was 2.525 billion tons. The world produced 877.9 million tons of maize, 734.9 million tons of rice, 671.4 million tons of wheat, and 240.9 million tons of soybeans (Figure 2) (FAO, 2014). These numbers will need to increase as the population increases, however there are environmental and economic issues that come with the increasing demand for food.

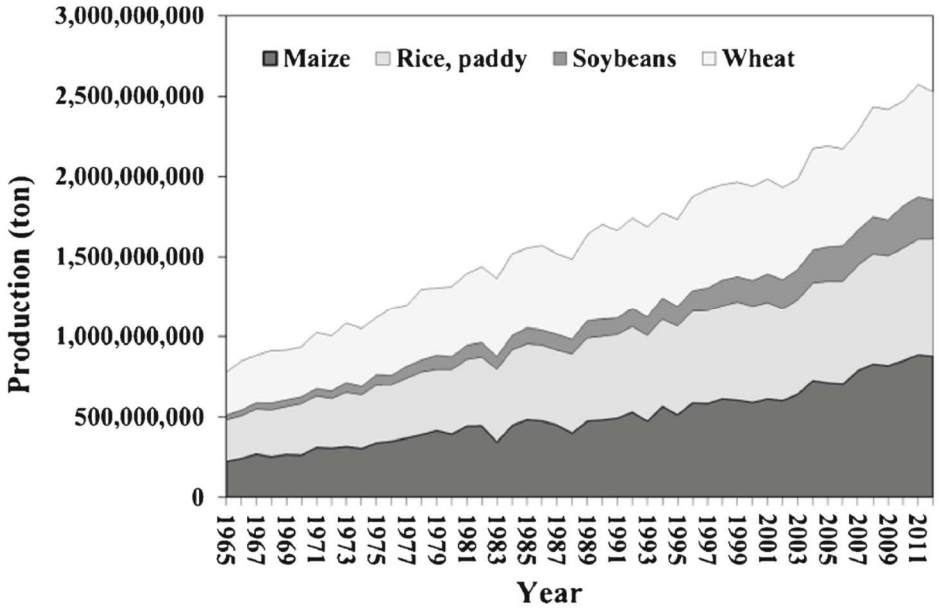


Figure 2 World crop production for maize, rice, soybeans and wheat in tons from the (FAO, 2014).

Issues with Increasing Production

To increase food production, we will either need to expand geographically and use more land or increase the intensity of farming practices on the current production areas. However, as the world population increases, so does the land used for urban areas. Since the 1960's, the "green revolution", there has been an intensification of farming which has caused a decrease in usable agriculture land. This loss of land is due to soil erosion, decreased soil fertility, salinization, chemical build-up, and desertification of soils (Carvalho, 2006). The cost of producing more arable land is high, due to the loss of important ecosystems through deforestation (Alexandratos, 1999). The "green revolution" was the beginning of the massive increase in fertilizer usage. Nitrogen, phosphorus, and potassium (N, P, K) were applied in quantities never used before, as well as a push for increased irrigation. The amount of phosphorus fertilizer

used in China has increased over 100-fold between 1960 (0.05 Mt) and 2010 (5.3 Mt) (Lang, Christie, Zhang, & Li, 2018).These new exhaustive farming practices were able to increase crop production world-wide, particularly on the high producing crops such as corn and wheat (Carvalho, 2006). However, now that it has almost been 60 years since the "green revolution" we are in need of new innovative ways that can increase crop production, while at the same time, preventing the negative impacts on the land that is cultivating the needed food supply for the growing population.

There are several reasons why simply increasing mineral fertilizers is not a positive economic or environmental solution to increase crop production. First of all, the supply of phosphorus reserves is estimated to be depleted in 100 year (Steen, 1998). We will then be forced to resort to the low-quality reserves which take additional processing and results in a greater amount of waste that will need to be disposed of, increasing P fertilizer costs (Cordell et al., 2009). However, it has been estimated that we could delay the P depletion date by over 20 years, if the mining and agriculture industries find more efficient ways of managing the P demand (Lang et al., 2018). When considering natural finite non-renewable resources, there always comes a time that we reach a maximum rate or 'peak' of consumption, and at that point production begins to decline. Based on conservative industry data from the US Geological Survey, the European Fertilizer Manufactures Association, and the International Fertilizer Industry Association, the 'peak' phosphorus fertilizer year was calculated to be 2033 (Figure 3) (Cordell et al., 2009). The world production of phosphate rock will hit its maximum and

begin to decline in quality and accessibility, meaning the price will increase and the quality will decrease (Jasinski, 2006).

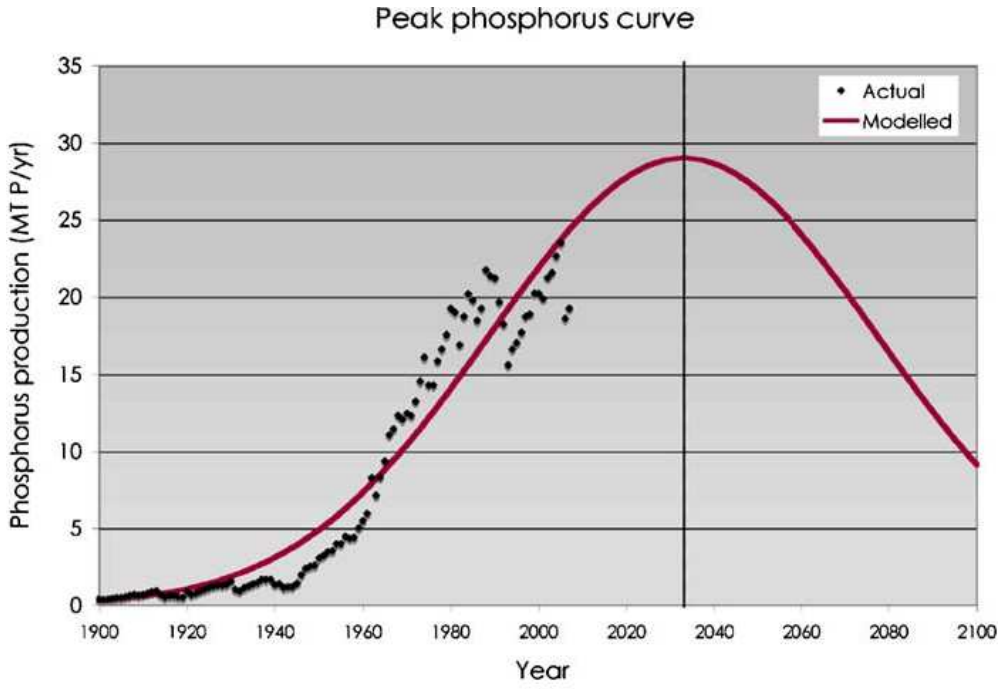


Figure 3Indicative peak phosphorus curve, illustrating that, in a similar way to oil, global phosphorus reserves are also likely to peak after which production will be significantly reduced (Cordell, Drangert, & White, 2009).

Soluble P fertilizer such as phosphate and super phosphate fertilizers that are produced from insoluble rock phosphate and phosphoric acid cause environmental concern due to heavy metal contaminates (Rutherford, Dudas, & Arocena, 1995). This is due to the fact that phosphate rock (PR) contains minor metal constituents in the ores such as; arsenic (As), chromium (Cr), lead (Pb), mercury (Hg), nickel (Ni), vanadium (V), and cadmium (Cd). Heavy metal build-up in the soil is a real concern due to the residual metal contaminates that are carried over from the production process, contaminate the P fertilizer, and get applied year after year to the soil. There are several large PR deposits world-wide, and heavy metal concentrations have been analyzed for the main deposits. Table 1 illustrates the heavy metal concentrations in 91% of the worlds

phosphate rock deposits in 1992, as well as the estimated amount that is added to the soil at a given P fertilizer rate (20 kg P/ha) (Mortvedt, 1996).

Table 1. Average heavy metal concentrations in phosphate rock deposits and estimated inputs to soils by P fertilizers (Mortvedt, 1996).

	Heavy metal concentration, mg/kg						
PR deposit	As	Cd	Cr	Pb	Hg	Ni	V
Russia	1	0.1	13	3	0.01	2	100
USA	12	11	109	12	0.05	37	82
South Africa	6	0.2	1	35	0.06	35	3
Morocco	11	30	225	7	0.04	26	87
North Africa	15	60	105	6	0.05	33	300
Middle East	6	9	129	4	0.05	29	122
Avg of 91% of PR reserves	11	25	188	10	0.05	29	88
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mg/kg of P	71	165	1,226	66	0.29	189	578
g/ha/yr applied with 20kg P/ha	1	3.3	25	1	0.01	4	12
Tolerance limit in soil, mg/kg	2	100	100	2	50	50	300

Besides having an increase in heavy metal accumulation in the soil, mining phosphate mineral can pose other issues. The mining of phosphate is not an efficient process, due to the high amounts of gypsum that needed to be disposed of, as well as the hydrogen fluoride gas that is emitted as a highly volatile and poisonous gas (Sharma, Sayyed, Trivedi, & Gobi, 2013). Also, continual additions of P fertilizers can affect the microbial diversity and soil, which leads to the loss of soil fertility (Gyaneshwar, Kumar, Parekh, & Poole, 2002).

PLANT GROWTH PROMOTING BACTERIA

Due to the issues caused by harsh farming practices and the concern of the impacts to the environment, the science community is determined to find alternatives that are beneficial for producers, as well as safe for the environment. One of these

solutions is to research and expand biofertilizer and plant growth promoting bacteria (PGPB) technologies. We are now trying to take advantage of the relationship that is formed between the plant and microbes. PGPB are bacteria that are free living in the rhizosphere, on the phyllosphere, and even endophytes that live within the plant (de Souza, Ambrosini, & Passaglia, 2015). Endophytic bacteria have been found in root, stem, leaf tissues, and seeds of plants. There is an abundance of endophytes in the roots compared to other locations within the plant (Kiani et al., 2019). The genetics of the plant are a determining factor in the microbial communities within the plant, such that bacterial endophytes isolated from conventional and transgenic soybeans had statistically different microbial communities (de Almeida Lopes et al., 2018). Also, the diversity of the endophytes can be considered a subset of the bacteria that reside in the rhizosphere (Marquez-Santacruz, Hernandez-Leon, Orozco-Mosqueda, Velazquez-Sepulveda, & Santoyo, 2010). There are distinct genomic differences between endophytic bacteria and ones that live freely in the rhizosphere, however the exact genes that are involved in this difference are still under study (S. Ali, Duan, Charles, & Glick, 2014). Root cracks typically leak plant metabolites that draw additional bacteria in, causing it to be a point of common entry, in addition to tissue growth wounds (Hallmann, QuadtHallmann, Mahaffee, & Kloepper, 1997; Sprent & Defaria, 1988). Bacteria can enter plant through the stomata, the lenticels (Scott, Chard, Hocart, Lennard, & Graham, 1996), the germination radicle (Gagne, Richard, Rousseau, & Antoun, 1987), newly formed lateral roots and root hairs (Huang, 1986), and even through an intact epidermis layer by hydrolyzing the cell wall (Hallmann et al., 1997).

PGPB have the ability to improve plant growth and protect from stresses (Grover, Ali, Sandhya, Rasul, & Venkateswarlu, 2011). Some of the benefits of PGPB are biological nitrogen fixation (Sorkhoh et al., 2010), mitigating stress due to production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase to lower plant ethylene levels (Zhou et al., 2019), production of indole-3-acetic acid (Gusain, Kamal, Mehta, Singh, & Sharma, 2015), phytohormones (Amara, Khalid, & Hayat, 2015), siderophore production (Maglangit, Tong, Jaspars, Kyeremeh, & Deng, 2019), heavy metal immobilization (Maity, Chen, Huang, Sun, & Chen), salt tolerance (Li & Jiang, 2017), disease suppression (Korejo et al., 2019), phosphate solubilization (Marra, de Oliveira-Longatti, Soares, Olivares, & Moreira, 2019) and many more. PGPB have been found in the following species; *Pseudomonas*, *Mesorhizobium* (Valverde et al., 2006), *Rhizobium, Bacillus* (Elkoca, Kantar, & Sahin, 2008), *Burkholderia* (Ciccillo et al., 2002), *Mycobacterium* (Egamberdiyeva, 2007), *Azospirillum* (Hungria, Nogueira, & Araujo, 2013), *Gluconacetobacter* (Beneduzi et al., 2013), and *Arthrobacter* (Upadhyay, Singh, Saxena, & Singh, 2012).

Biological Nitrogen Fixation

The atmosphere is made up of 78% nitrogen (N), however it is in a form that is not directly available to plants and animals. The nitrogen in the atmosphere is in the N_2 form with a strong triple bond that hold the two nitrogen atoms together (Fields, 2004). Due to the high need that plants have for nitrogen, large amounts of fertilizer are used to supply the N in a form that the plants can utilize. Unfortunately, N can be easily converted to atmospheric greenhouse gases (Flechard et al., 2007), or run-off water and

accumulated in ground water (Trindade, Coutinho, Jarvis, & Moreira, 2001). However, a symbiotic relationship between legumes and rhizobia has the ability to fix N_2 from the atmosphere and break the triple bond to make it available to the plants. Rhizobia form nodules on legumes and utilize an enzyme called nitrogenase that can convert N_2 into ammonia (Postgate, 1982). In return the rhizobia receives carbon from the plant's photosynthates (Howard & Rees, 1996).

Inoculating maize with nitrogen-fixing bacteria was able to increase plant growth, as well as increase the levels of N by 0.30 - 0.82 g N/plant. Inoculated plants significantly changed the diazotrophic community and ammonia oxidizers in the rhizosphere (Ke et al., 2019). Several factors can play a role in the biodiversity and N-fixing abilities of microbes in the rhizosphere. Soil temperatures need to be in a moderate range to form nodules and for the nitrogenase enzyme to function properly (Roughley & Dart, 1969). The level of soil mineral N in the rhizosphere needs to be low for the bacteria to put energy into utilizing the nitrogenase enzyme, because it uses less energy to absorb soil available N (Cannell & Thornley, 2000). As plants get larger and older the diversity of the rhizobia associated with their rhizosphere increases (Dinnage et al., 2019). Also, soil water is a critical component for nitrogen fixation efficiency due to less nodulation and gas permeability. When the water level in the soil went from fully saturated to two-thirds water capacity the amount of N fixed decreased by 18-40%, and when the water was decreased even further to one-third capacity the N uptake decreased by 44-69% (Pimratch et al., 2008).

It is well documented that ammonia and oxygen are signal regulators of nitrogen fixation at the *nif* gene transcript level (Shi et al., 2016). Through genomic comparative analysis nine genes (*nifB, nifH, nifD, nifK, knife, nifN, nifX, hesA, nifV*) were identified that form a compact *nif* cluster that encodes the nitrogenase enzyme in *Paenibacillus* strains (Xie et al., 2014). In many cases, N-fixing bacteria utilize *nifA* as the positive transcriptional regulator and *nifL* as the negative transcriptional regulator (Hill, Austin, Eydmann, Jones, & Dixon, 1996). Some *nif* genes can be found on the chromosomes of N-fixing bacteria, while the majority of *nif* genes are located on plasmids (Zamorano-Sanchez et al., 2012).

Indole-3-acetic acid

Indole-3-acetic acid (IAA) is the most common natural auxin phytohormone. IAA can be produced by rhizosphere, endophytic, and epiphytic bacteria, as well as plants, fungi, and cyanobacteria (Duca, Lorv, Patten, Rose, & Glick, 2014). Different genera such as *Pseudomonas* (Patten & Glick, 2002), *Pantoea* (Kulkarni, Nayak, Sajjan, Oblesha, & Karegoudar, 2013), *Rhizobium* (Defez et al., 2017), *Azospirillum* (Xie, Xu, Zhao, & Chen, 2005), *Enterobacter* (Ghosh, Sen, & Maiti, 2015), *Klebsiella* (Gang, Sharma, Saraf, Buck, & Schumacher, 2019), and *Streptomyces* (Tuomi, Laakso, & Rosenqvist, 1994) can affect plant growth through IAA production. IAA can enhance plant growth, protect plants from environmental stresses, signal to the plant to avoid shade (Tao et al., 2008), and has the ability to control plant development such as cell division, elongation, fruit development and senescence (Phillips et al., 2011), initiate root, leaves, and flowering (McSteen, 2010), change the number and length of lateral and primary roots, stimulate

root hair formation, vascular development, secondary wall thickness, and increase xylem cell size (Uggla, Moritz, Sandberg, & Sundberg, 1996).

IAA is produced by utilizing tryptophan and other root exudate molecules (Kang et al., 2019). There are three main IAA producing pathways; indole-3-pyruvic acid (IPA), indole-3-acetamide and indole-3-acetonitrile (Duca et al., 2014). Plants have a beneficial growth response from the appropriate level of IAA, however, if the plant receives excess levels it can slow the growth of the plant (Beyeler, Keel, Michaux, & Haas, 1999). Plants have a regulatory system that helps to minimize the effects of IAA overproduction. Free IAA is inactivated within transgenic tobacco plants by conjugation to sugar, amino acids, or peptides (Sitbon, Sundberg, Olsson, & Sandberg, 1991). Different strains of plants can handle different levels of IAA, and varying strains of bacteria produce varying levels of IAA (Shokri & Emtiazi, 2010). The IAA biosynthesis changes based on certain environmental conditions such as pH, temperature, carbon and nitrogen sources (Chandra, Askari, & Kumari, 2018), and the presence of tryptophan, vitamins, salt, and oxygen (Apine & Jadhav, 2011).

Determining the molecular regulations at the genomic level of IAA is an in-depth process that is still being elucidated. Due to multiple pathways of IAA biosynthesis, bacteria having the capability to use more than one pathway, and whether the genes are located on a chromosomal or on a plasmid are some of the reasons it is difficult. Due to the molecular depth of the pathways the majority of the research has been conducted on the IPA pathway (Duca et al., 2014). Indole-3-pyruvate decarboxylase (IPDC), a key enzyme in the IPA synthetic pathway, is encoded by the *ipdC* gene (Phi,

Park, Ryu, Park, & Ghim, 2008). By using *tyrR*, a regulation protein of aromatic amino acids such as tryptophan, and *ipdC* mutant strains it was found that both TyrR and IPDC are critical for the biosynthesis of IAA in *Enterobacter cloacae* (Ryu & Patten, 2008). One of the most studied genes is IAA-lysine synthetase gene (*iaaL*) which converts IAA into IAA-lysine (Glass & Kosuge, 1988). The encoded protein plays a key role in the amount of free IAA that is available. Some additional putative genes utilized in the degradative effects of IAA are; beta-oxoacyl-CoA thiolase (*iaaA*), a CoA ligase acting on aromatic acids (*iaaB*), a hydantoinor 5-oxoproline hydrolase (*iaaCE*), an acyl-CoA dehydrogenase (*iaaF*), a coenzyme B12-dependent mutase (*iaaGH*), a molybdenum enzyme of the xanthine dehydrogenase family (*iaaIJK*), and a periplasmic binding protein for an ABC transporter system (*iaaM*) (Duca et al., 2014).

Suppression of fungal pathogens

Fungal suppression is a critical component of food production, considering the vast amount of food lost due to phytopathogens. Genera such as *Bipolaris* and *Curvularia* have a USD $10 billion economic impact worldwide due to the severe diseases they cause in plants and animals (Bengyella et al., 2019). *Fusarium* head blight (FHB) is one of the most destructive disease on wheat in China and other wheat producing countries (Wegulo, Baenziger, Nopsa, Bockus, & Hallen-Adams, 2015). *Colletotrichum capsica* is a fungal pathogen that accounts for 10-60% of the yield losses on chili, *Capsicum annuum*, in parts of India. Additionally, there are risks that pesticides have on humans and the environment, such as toxicities to plants and animals, skin irritation, birth defects, tumor formation, genetic changes, blood and nerve disorders,

endocrine and reproduction disruptions (Lorenz, 2017). One alternative to the harsh fungicide chemicals are biological control agents (BCA), organisms that have the ability to suppress the pests such as pathogenic fungi.

Antibiosis is the communication between two organisms, typically through diffusion of complex molecules, that results in an antagonistic effect on one of the organisms. One of the most common examples of this is antibiotics to suppress microbial infections. There is a wide variety of chemical compounds as well as varying modes of action of antibiotics produced by bacteria. Some commonly researched compounds and mechanisms are hydrogen cyanide (cellular respiration), penicillin and butyric acid (cell wall synthesis), phenylethanol (transport systems), and hydrolytic enzymes, cyclic lipopeptides, and polymyxin B (impair cell membrane integrity) (Frey-Klett et al., 2011). More specifically, hydrolytic enzymes such as glucanase are produced by bacteria, and have a significant impact on the growth of pathogenic fungi such as *Fusarium moniliforme* (45–56%) and *Colletotrichum falcatum* (52–63%), *Fusarium oxysporum* (58–63%), *Rhizoctonia solani* (42–53%) and *Macrophomina phaseolina* (53–61%) (Zia et al., 2019). Antifungal cyclic lipopeptides, such as iturins and surfactins, were shown to suppress fungal growth and maintain grape quality (Zhang et al., 2019). Propionic (0.5%), benzoic (0.05%), and sorbic acid (0.1%) have fungal inhibitory activity on *Aspergillus flavus* (Moon, Kim, Chun, & Lee, 2018). Beyond the direct bacterial-fungal interactions, fungal suppression can be induced by interacting with the plant's defense system.

In 1991, it was discovered that beneficial microorganisms can elicit induced systemic resistance (ISR) and systemic acquired resistance (SAR) of the plant (Alstrom, 1991; Vanpeer, Niemann, & Schippers, 1991). These systemic resistance pathways have an effect on a broad range of pathogens and have been reported in many different plants (Ton, Van Pelt, Van Loon, & Pieterse, 2002). ISR is activated when non-pathogenic rhizobacteria, or their chemical signals, come in contact with the root system and activate a state of resistance in the plant. When plants come in contact with these inducers, the plant systematically responds and triggers distal defense mechanisms (Walters, Ratsep, & Havis, 2013). SAR is most commonly activated by a pathogen encounter in the rhizosphere and is typically longer lasting (Shine, Xiao, Kachroo, & Kachroo, 2019). When ISR is triggered, a significant proportion of genes involved in phytohormone signaling are upregulated such as jasmonic acid and ethylene (Sharma, Chen, Navathe, Chand, & Pandey, 2019). Some potential mechanisms of the ISR jasmonic acid or ethylene triggered pathways are; an increase in the endogenous 12-oxophytodienoic acid, SA, and flavonol levels (Krol, Igielski, Pollmann, & Kepczynska, 2015), mitogen-activated protein kinases (a signaling cascade) (Beckers et al., 2009), and production of phytoalexin at the sight of contact (Ahn, Lee, & Suh, 2007). SAR typically is initiated by the signaling of salicylic acid (SA), which triggers pathogenesis-related (PR) genes, encoding the PR antimicrobial activity proteins (Ali et al., 2017). Some plant responses to the SA-signaling pathway are an increase in reactive oxygen species, and programmed cell death (PCD), potentially limiting the water and nutrients that the pathogen would be able to receive from the host (Heath, 2000).

Culturing, Characterizing, and Commercializing PGPB

Due to the vast benefits rhizosphere and endophytic bacteria have on plant growth, researchers and industry have been trying to find innovative ways of harnessing their benefits for food production and to minimize the negative effects conventional farming has on the environment. However, based on 16S rRNA sequencing, it is estimated that 99% of soil bacteria are unculturable (McCaig, Grayston, Prosser, & Glover, 2001). With the use of different media and growing conditions we were able to reduce that number. Due to the high demand for furthering our knowledge and commercialization of PGPB, novel culturing methods and culture-independent techniques have helped to characterize bacteria.

Modifying the growth media, in particular by diluting specific nutrients has increased the number of culturable soil oligotrophic bacteria (Vartoukian, Palmer, & Wade, 2010). An additional approach has been to modify the growing conditions such as increasing the inoculation time, or varying the oxygen levels, temperature, and pH (Janssen, 2008). Researchers have better replicated the native growing conditions by utilizing community culturing (Armanhi et al., 2018), and co-culturing (Slaughter & Cadet, 2018). Another tool to help identify and characterize PGPB is matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) (Avanzi et al., 2017). Identification of bacteria is based on the bacterium spectrum and then compared to the database containing peptide mass fingerprints (Singhal, Kumar, Kanaujia, & Virdi, 2015). Additionally, chemotaxonomic phenotype comparisons can help identify and characterize isolates for commercialization. This can be accomplished

through testing the isolates metabolic capabilities, which has been made more readily possible by comparison tools such as Biomerieux API and Biolog Gen III MicroPlate test systems (Adley & Saieb, 2005; Hamedo, 2016).The Gen III Micro Plates helps characterize the metabolic capabilities by utilizing a 96 well plate that contains a positive and negative control, 23 chemical sensitivity assays, and 71 carbon source assays. This process gives you a phenotypic fingerprint making it possible to compare chemotaxonomic similarities (Wragg, Randall, & Whatmore, 2014).

Culture-independent approaches have vitalized microbial ecology and phylogenetics. Since 1977 when Carl Woese pioneered the use of 16S rRNA, the gene sequence identity has been utilized as a base line of new species (Woese, 1987). Due to the vast 16S rRNA sequencing database, we can utilize the similarity threshold of 97% to distinguish between different operational taxonomic units (OTU) (Stackebrandt & Goebel, 1994). Full genome sequencing has taken identification and characterization to an immensely deeper level. Full genome sequences give you the ability to search for specific putative genes, and conduct genome comparison assays. When attempting to identify an isolate, genome comparison tests such as amino acid identity (AAI) (M. et al., 2018), average nucleotide identity (ANI) (Yoon, Ha, Lim, Kwon, & Chun, 2017), and genome-to-genome-distance-calculations (GGDC) (Meier-Kolthoff, Auch, Klenk, & Goker, 2013) are utilized.

The use of PGPB bioinoculants has grown immensely over the last decade, and expanded beyond biofertilizers which have been commercialized over a century ago (Nehra & Choudhary, 2015). The benefits of PGPB previously stated, have more recently

be researched on larger field trial scales. For example, *Jeotgalicoccus huakuii* NBRI 13E showed benefits in mitigating salt stressed soils, and when the isolate was evaluated on vegetables in the field it significantly enhanced the crop health parameters and yield, compared to the respective control (Misra, Dixit, Mishra, & Chauhan, 2019). *Bacillus* RM-2 showed many beneficial parameters in the laboratory such as P solubilization, ACC deaminase activity, antifungal activity, IAA production and ammonia production. When tested in the field RM-2 treated plants had significantly greater yield parameters such as number of pods and grain yield per plant than the control plants (Minaxi, Nain, Yadav, & Saxena, 2012). A multi-strain biofertilizer, composed of novel strains of *Mesorhizobium cicri*, *Pseudomonas* sp. and *Bacillus* sp., was tested on four fields, with three replications each. It was found that the combination of the multi-strain biofertilizer and manure was the ideal treatment for chickpea based on increases in nodules, plant growth, yield, and nutrient uptake (Ahamd et al., 2017)

PHOSPHATE SOLUBILIZING BACTERIA

Soil Phosphorus Forms

The second most vital macronutrient for plant production is phosphorus (P). Phosphorus is needed in most all life processes such as respiration, photosynthesis, energy transfer and storage, signal transduction, cell division and elongation, root and seed formation, nitrogen fixation, and more (Sashidhar & Podile, 2010; Zaidi, Khan, Ahemad, & Oves, 2009). When crops are unable to acquire phosphorus from the soil the plant health and ultimately yield are compromised. It is said that more than 40% of the

world's arable land has compromised production due to phosphorus plant deficiencies (Balemi & Negisho, 2012). Due to this, and the important role P plays in plant production, large amounts of fertilizers are used to maximize yield potential. Unfortunately, up to 80% of the 15 million tons of P fertilizer applied worldwide each year is lost due to rapid immobilization (Gyaneshwar et al., 2002). In addition to the low P fertilizer use efficiency, it also causes environmental issues, and is a very costly portion of crop production (Mahanta et al., 2018). In 1993 Goldstein, Rogers, and Mead estimated that it costs $4 billion annually to produce enough energy to produce phosphatic fertilizer to meet the world's needs (Goldstein, Rogers, Mead, 1993).

There is an adequate amounts of phosphorus in the soil, 400-1200 ppm, however less than 1.0 ppm of P is in a soluble form (Goldstein, 1999; Rodriguez & Fraga, 1999). Low availability is due to adsorption to soil particles, minerals, or P incorporated ions into soil organic matter (Holtan, Kamp-Nielson, & Stuanes, 1988). The forms of phosphate that a plant can assimilate, are negatively charged molecules, typically HPO_4^{2-} or H_2PO^- (Beever & Burns, 1980). In both undisturbed and cultivated soils, P is typically near the surface of the soil, due to the cycling of P through vegetation and decomposition, as well as fertilizer applications (Menzies, 2009). It is estimated that only 1% of the total soil P in the top 30 cm is assimilated into living plants each growing season. This suggests that there would be enough soil P to sustain maximum crop yields worldwide for 100 years, without any additional inputs, if soil P could be solubilized (Khan, Zaidi, & Wani, 2007).

Soil phosphorus is in three main forms: soil P solution, labile P, and non-labile P (Figure 4) (Menzies, 2009). It fluctuates between these three phases to stay at a fairly constant ratio, which is typically pre-determined by the parent material as well as the plant and microbial P demand. The labile inorganic P is most commonly in an adsorbed orthophosphate form. The adsorbed orthophosphate is able to buffer the soil P solution when there is a concentration change, to ensure an equilibrium with the soluble P pool. This predominantly happens when plants take up phosphate which then causes a concentration change, thus desorbing P from the labile phase into the soil solution. The opposite occurs when soluble fertilizer is applied or when P is mineralized into solution, thus P is once again adsorbed from solution to maintain equilibrium (Menzies, 2009).

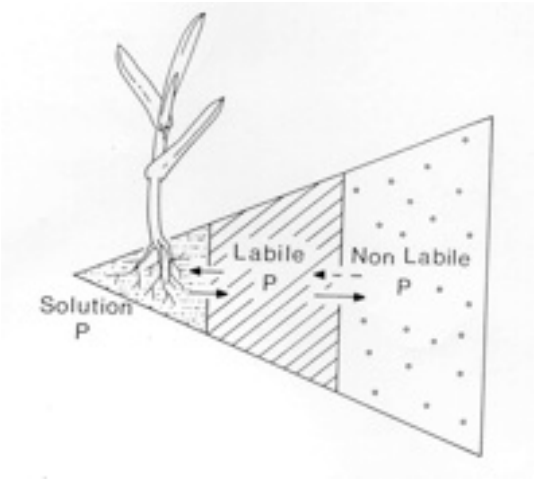

Figure 4. Schematic representation of the forms of phosphorus present in soil (Menzies, 2009).

Unlike N, plants do not receive their P from the atmosphere. Phosphorus has a 'sedimentary' cycle, meaning it has a water-soluble phase and a rock or sedimentary phase (Begon, Harper, & Townsend, 1990). Its biogeochemical cycling is mainly centered around microorganisms, by means of redox reactions. Through the transferring of

electrons, P can be found in a range of oxidative states, from (-3) phosphine to (+5) phosphate (Ohtake et al., 1996). At the most basic level, phosphorus can exist in two main forms in the soil, inorganic and organic compounds (Khan, Almas, & Ees, 2014). We will look deeper into each of these categories of soil P.

Inorganic P Compounds

Phosphorus reserves are found in phosphate rock (PR) and other deposits, which is insoluble in this form. These primary minerals in the soil are found in stratum rock, such as apatite, hydroxyapatite, and oxyapatite (Rodriguez & Fraga, 1999). The areas with the largest mining production of sedimentary phosphate rock are in Africa, Morocco and the Western Sahara, as well as in China, and the United States (Table 2). It has also been found that the Atlantic and Pacific Oceans have large P reserves on the continental shelves and the seamounts (Rodriguez & Fraga, 1999).

Table 2. Locations of phosphate deposits throughout the world in 2016 and 2017 (Zinke, 2018).

	Mine production		Reserves[4]
	2016	2017[e]	
United States	27,100	27,700	1,000,000
Algeria	1,270	1,300	2,200,000
Australia	3,000	3,000	[5]1,100,000
Brazil	5,200	5,500	1,700,000
China[6]	135,000	140,000	3,300,000
Egypt	5,000	5,000	1,300,000
Finland	940	950	1,000,000
India	2,000	1,800	65,000
Israel	3,950	4,000	74,000
Jordan	7,990	8,200	1,300,000
Kazakhstan	1,500	1,600	260,000
Mexico	1,700	2,000	30,000
Morocco and Western Sahara	26,900	27,000	50,000,000
Peru	3,850	3,900	400,000
Russia	12,400	12,500	700,000
Saudi Arabia	4,200	4,500	1,400,000
Senegal	2,200	2,200	50,000
South Africa	1,700	1,800	1,500,000
Syria	—	100	1,800,000
Togo	850	1,000	30,000
Tunisia	3,660	3,700	100,000
Vietnam	2,800	3,000	30,000
Other countries	1,950	1,940	900,000
World total (rounded)	255,000	263,000	70,000,000

[1] Defined as phosphate rock used by producers + imports – exports.
[2] Marketable phosphate rock, weighted value, all grades.
[3] Defined as imports – exports + adjustments for industry stock changes.
[4] See Appendix C for "reserve" definitions and information concerning data sources.
[5] For Australia, Joint Ore Reserves Committee- compliant reserves were about 290 million tons.
[6] Production data for large mines only, as reported by National Bureau of Statistics of China.

Besides P in primary rock deposits, it can also form a complex with secondary minerals. The hydrated oxides on the surfaces of manganese (Mn), aluminum (Al), iron (Fe), and calcium (Ca) can associate with mineral P, these forms of phosphorus are poorly soluble. The soil pH and soil mineral type and concentration play an important role in the fixation and precipitation of P in the soil (Khan et al., 2014). When the soil pH is not neutral, P will be fixed by free oxides and hydroxides of the previously stated minerals. When this happens, the soil phosphorus will form highly stable compounds and become unavailable for the plant. For example, in an alkaline soil environment P forms a strong complex with Ca and in an acidic environment P will form complexes with Al, Fe, and Mn (A. H. Goldstein, 1986). Table 3 shows many of the different forms of phosphorus minerals found in the soil.

Table 3. Forms of phosphorus minerals found in the soil (Khan et al., 2014).

Soil Conditions	Mineral	Chemical Formula
Acidic soils	Strengite	$FePO_4{*}2H_2O$
Acidic soils	Variscite	$AlPO_4{*}2H_2O$
Neutral and calcareous soils	B-tricalcium phosphate	$Ca_3(PO4)_2$
Neutral and calcareous soils	Dicalcium phosphate	$CaHPO_4$
Neutral and calcareous soils	Dicalcium phosphate dihydrate	$CaHPO_4{*}2H_2O$
Neutral and calcareous soils	Fluorapatie	$Ca_5(PO4)_3$ F
Neutral and calcareous soils	Hydroxyapatie	$Ca_5(PO4)_3$ OH
Neutral and calcareous soils	Octacalcium phosphate	$Ca_4H(PO4)_3{*}2\text{-}5H_2O$

Organic P Compounds

The second source of soil phosphorus is the organic component of soil P. It has been estimated that 30-50% of the total soil P is in the organic form, however there are extreme cases and it can range from 4-90% (Yadav & Verma, 2012). These organic labile pools are typically produced by both microorganisms and plants. The most stable and abundant form of organophosphorus is phytate, also called inositol phosphate, which is formed by a series of phosphate esters ranging from monophosphates up to hexaphosphates, and it accounts for approximately 10-50% of the total organic P (Khan et al., 2014). Some other key organic sources of P are sugar phosphates, nucleotides (0.2-2.5%), phosphoprotein (trace amounts), phosphonates, and phospholipids (1-5%). Organophosphates cannot be utilized by the plant directly because most of these P sources are high molecular weight compounds (Khan et al., 2014; Yadav & Verma, 2012). They need to be first converted to either low molecular weight organic phosphate or soluble ionic orthophosphate (Pi). Both of these transformations typically

happen by biological conversion, because most these compounds are resistant to chemical hydrolysis (Goldstein, 1999).

However, besides these previously stated organophosphates, xenobiotic phosphonates are present in the soil. The sources of these C-P compounds are from pesticides, detergent additives, antibiotics, and flame retardants. These synthetic chemicals that are released into the soil environment are not easily hydrolyzed and decomposed. However, there have been some studies that show that these P sources can be solubilized by microbes (McGrath, Hammerschmidt, & Quinn, 1998).

When microbes are able to convert P from an insoluble organic form to a soluble form this is considered mineralization. This process is similar to the mineralization of N however, when P is mineralized and added to the soil solution it almost immediately is bound to inorganic soil minerals, whereas N is able to stay in the labile soil pool. Due to this differentiation, it makes measuring mineralized P difficult (Menzies, 2009).

Phosphate Solubilizing Microorganisms

Soil microorganisms play a key role in the soil phosphorus cycling. Typically, plants and microorganisms sequester phosphorus as the orthophosphate form, however, to transform the P from the non-labile insoluble form to the soil solution, phosphate solubilizing microorganisms are needed. Of the organisms, bacteria play one of the largest roles (Khan et al., 2007). The number of phosphate solubilizing bacteria in soils tend to out-number phosphate solubilizing fungi, by approximately 2-150 fold. It

has been estimated that 1-50% of the bacteria in the soil are PSB, and 0.1-0.5% of the total fungi are able to solubilize P (Gyaneshwar et al., 2002). PSB live both in the plant, as endophytes, or in the soil rhizosphere (Katznelson et al., 1962). By releasing root exudates, mucigels, and root cells, plants create an area of intense microbial activity and competition on the root-soil interface, the rhizosphere. This is also where most the PSB reside in the soil. There is a lot of competition for P in the rhizosphere, and bacteria have very efficient mechanisms for sequestering soil P (Table 4). Microbes hydrolyze insoluble inorganic and organic phosphorus sources through different solubilization and mineralization technics. There have been numerous studies over the years about the mechanisms P-solubilizing organisms are able to use to solubilize/mineralize the P, however there is still some uncertainty of how the organisms are able to solubilize the phosphorus. When P is solubilized or mineralization by PSB, it is also typically immobilized for a period of time. Bacteria are especially important when it comes to storing P by solubilizing then immobilizing the soil P by assimilating it into their cells as a labile source. The sequestering and release of the P by the cells is influenced by physico-chemical changes in the environment (Khan et al., 2014).

Table 4. The potential orthophosphate uptake rate by soil organisms (Menzies, 2009).

	Estimated fresh weight of biomass in the surface 10cm[a] (g/m^2)	Calculated P_i uptake rate at background conc of 10 µM[b] (µmol P/min)	Ratio of uptake relative to plant roots
Bacteria	103	359	9.0
Fungi	260	71	1.8
Plant roots	5000	40	1.0

[a] Bacterial and fungal biomass based on Clark and Paul assuming a dw/fw ratio of 0.2. Root biomass based on a value of 50 cm^{-2} for root length per unit volume and a conversion factor of I mg fw cm^{-1} root length.
[b] Calculated assuming all fungi behave like *A. nidulans*, bacteria like *E. coli*, and plant roots like millet.

Inorganic Phosphate Solubilizing

The most commonly accepted and studied theory for solubilization of inorganic P is that bacteria secret low molecular mass organic acids (OA) (Rodriguez & Fraga, 1999). These OA are able to chelate mineral ions, such as Ca, Al, or Fe and release P, and/or decrease the pH to bring P into solution (Pradhan & Sukla, 2005). The pH drop ultimately releases the P-ions from the mineral by H^+ substitution (Goldstein, 1999). Some of the most commonly measured organic acids are; gluconic, formic, malic, succinic, acetic, oxalic, citric, and lactic acid (Bakri, 2019; Topolska et al., 2013; Wei, Zhao, Shi, et al., 2018).

Gluconic acid is one of the most commonly researched organic acid by PSB (Goldstein, 1999; Rodriguez & Fraga, 1999). The low molecular weight organic acid, gluconic acid, is produced when glucose dehydrogenase and the cofactor pyrroloquinoline quinone, oxidizes glucose extracellularly and produces gluconate, the ester form of gluconic acid (Rodriguez & Fraga, 1999). When glucose is metabolized through extracellular oxidation gluconic acid and often 2-ketogluconic acid are produced in the periplasmic space. Once the OA is excreted from the cell, it is able to release the P from the complex, typically Ca, due to the acidic protons around the cell (Goldstein, 1999).Other OA have been found to have similar or additional impacts on solubilizing phosphate.

When phosphorus is present, even if it is in a soluble form, formic acid is produced (Chen, Yang, Zhang, & Wang, 2016). A compost pile was inoculated with PSB

and compared to an uninoculated pile; the highest produced OA by the PSB was formic acid. The formic acid concentration was 54.5 mg/g, 23 days after inoculation (Wei, Zhao, Shi, et al., 2018). When formic acid was measured in PVK medium containing 1.5% (w/v) glucose, the P-solubilization continued for 72h when it reached a maximum level of 687 mg/L (Mehta, Walia, Kakkar, & Shirkot, 2014). Organic acid production was measured for several different phosphate sources; tricalcium phosphate (TCP) Udaipur rock phosphate (URP), Mussoorie rock phosphate (MRP) and North Carolina rock phosphate (NCRP). The production of formic acid by *Acinetobacter rhizosphaerae*strain BIHB 723 was only detected when solubilizing TCP (Gulati et al., 2010). When nineteen PSB *Pseudomonas* strains were tested for organic acid production, half of the strains produced formic acid (Vyas & Gulati, 2009).

When pure organic acids are used to measure their effectiveness on TCP, URP, ferric phosphate (FP), and aluminum phosphate (AP); malic acid solubilizes the greatest amount of P from TCP, compared to five other OA (Gaind, 2016). Malic acid seems to be more beneficial in solubilizing zinc phosphate than tricalcium phosphate. When zinc phosphate was used as an inorganic P source, malic acid was the highest produced organic acid (Bakri, 2019). The impact of P deficiency and Al toxicity on organic acid production was measured, and malic acid secretion had a statistically positive correlation with oxalic and citric acid when Al toxicity and P deficiency were imposed (Barra et al., 2018). A rhizosphere PSB *Pseudomonas* was tested for organic acid production in correlation to phosphate solubilizing, several acids were identified but malic acid made up 34% of the total organic acid concentration (Zeng, Wu, & Wen,

2017). Six thermo-tolerant PSB isolates were tested for the production of seven different organic acids, and out of the seven acids, malic acid was the only OA produced by all six isolates (Yadav, Gothwal, Solanki, et al., 2015).

Pure organic acids were used to determine their effect on P-solubilization, and TCP, URP, FP, and AP were the tested P sources. Citric acid solubilizes the most amount of P from URP, FP, and AP compared to five other acids (Gaind, 2016). Citric acid is increased in the soil when P fertilizer, in the form of P_2O_5 is applied, and increase further when a PSB inoculum is added (Israr et al., 2016). When the organic acids produced from a *Bacillus* sp. isolated from phosphate mines, citric acid was the highest level of OA produced by the isolate (Yadav, Gothwal, Mathur, & Ghosh, 2015).

Lactic acid is able to help solubilize inorganic P by hydroxyl and carboxyl groups. Lactic acid production is stimulated when hydroxyapatite is supplemented in the media (Vassileva et al., 2010). Three *Bacillus* species and three alternative phosphorus sources; fish bone, poultry bone, and ash were compared for organic acid production to solubilizing the phosphorus in the raw material. Lactic acid was produced by all three *Bacillus* species, and from all tested doses of the phosphorus raw material, lactic acid was produced in the highest amount. Additionally, at higher doses of the raw materials gluconic acid production was decreased, however there were no detrimental effects on lactic acid production at higher rates of the raw material (Saeid, Prochownik, & Dobrowolska-Iwanek, 2018). However, when tricalcium phosphate is used as the P source, lactic acid is still produced but does not have a correlation to the microbial

biomass phosphorus, indicating it potentially is not adding in the phosphate sorption into the microbial cell (Wei, Zhao, Shi, et al., 2018).

When 100 ppm tricalcium phosphate was used as a phosphate source and five organic acids were produced by *Aspergillus niger*, succinic acid was produced at the highest level (Bakri, 2019). Out of 76 PSB isolated from agriculture soils, *Bacillus megaterium* was the dominant P-solubilizing bacteria, and succinic acid was the main acid produced. Four of the *Bacillus* strains were tested for OA production and succinic acid had a significantly greater amount than the other acids measured. There was a positive linear correlation between the amount of P-solubilized and the amount of succinic acid released (Zheng et al., 2018)

Also, inorganic acid is another possible mechanism used by P-solubilizing organism. These are typically produced by chemoautotrophs and some examples are HCl, nitric acid, carbonic acid, and hydrogen sulfide (H_2S) (Khan & Zaidi, 2007). For example, H_2S is able to react with the phosphate ion, complexed with a mineral such as iron, and produce ferrous sulphate, which in turn releases P (Park, Lee, & Son, 2009). Another possible mechanism to solubilize phosphate is the H^+ excretion during nitrification which originates from NH_4^+ resulting in an acidification of the microsite, solubilizing P (Park et al., 2009). Siderophores, which chelate ions such as iron, have also been tested as possible solubilizing agents (Hamdali et al., 2008). Also, indirectly through microbial respiration, H^+ ions are released through the dissolution of carbonic acid (H_2CO_3), due to the protons, P is solubilized from its molecule (Kim, McDonald, & Jordan, 1997; Zhu, Li, & Whelan, 2018). There are many possible paths of solubilizing P

and these are all being further investigated to determine the true mechanisms behind phosphate solubilization.

Genetics of Inorganic Phosphate Solubilization

The majority of all genetic studies on organic acids that solubilize phosphorus are conducted on gluconic acid. There has been some initial studies on the genes that play a role in the conversion of glucose to gluconic acid, and the majority of all the work was studied on *Pseudomonas* spp. (del Castillo, Duque, & Ramos, 2008; Fuhrer, Fischer, & Sauer, 2005). Lessie and Phibbs in 1984 referenced two main membrane bound enzymes in the glucose metabolism pathway; the glucose dehydrogenase and gluconate dehydrogenase, which oxidize the metabolism of glucose extracellularly. The respective genes for glucose dehydrogenase and gluconate dehydrogenase are (*gcd*) and (*gad*) (Miller et al., 2010). The (*gad*) gene resides in the gene cluster (*pqqA-F*) along with the cofactor pyrroloquinoline quinone (*pqq*) (Choi et al., 2008).

The ability of several *Pseudomonas* spp. to solubilize $Ca_3(PO_4)_2$ was researched and which organic acids were produced. The production of gluconic acid and 2-ketogluconic acid varied between the species. After further investigation, they found that the genomic organization for the subsequent genes (*pqqA-F*) and (*gcd*) varied by species. Some species had also additional copies of selected (*pqq*) genes and others had only one copy. Transposon mutants of the genes used to metabolize glucose to gluconic acid and 2-ketogluconic acid were used to determine which genes were critical for phosphate solubilization. Mutants were screened by measuring the reduction in the

$Ca_3(PO_4)_2$ solubilization ability. It was determined that the genes (*gcd*) and (*pqqE*) were critical for phosphate solubilization (Miller et al., 2010).

Soluble phosphate may negatively regulate the (*gcd*) gene transcription, due to the promoter of the gene that is negatively regulated by the cyclic AMP signal transduction system. This represses the production of gluconic acid and ultimately the ability to solubilize phosphate, by the upstream signal transduction system (Yamada, Asaoka, Saier, & Yamada, 1993). Six different levels of soluble phosphate on *Pseudomonas frederiksbergensis* JW-SD2 were tested. They measured the phosphate solubilization, the organic acid secretion, and growth of the microbes at each of the six levels. As the soluble phosphate levels increased the phosphate solubilization decrease, the gluconic, tartaric, and oxalic acids were decreased, and the growth increased. By cloning the (*gcd*) gene from *P. frederiksbergebsis* JW-SD2 they found that as the soluble phosphate levels increased, the expression of the gene was repressed. They predict that the gene is transcriptionally regulated by the level of soluble phosphate, which ultimately repressed the gluconic acid secretion and regulates the amount of phosphate that is solubilized (Zeng, Wu, & Wen, 2016).

It has been difficult to find a homologous gene that could be used to help identify a universal selector of a PSB based on OA production. Most the genetic work has been focused on gluconic acid alone and not the whole list of possible acids. However, the (*ppq)* cofactor was examined as a potential important regulator gene in inorganic P solubilization through organic acid release. Due to this, long-term nitrogen fertilized soils were tested with both the 16S rRNA gene sequencing and the (*pqqC)*

gene. The values were quite similar, however, the (*pqqC)* gene abundance tend to be consistently lower than the 16S rRNA abundance (Zheng, Hao, et al., 2017). This is a step towards a more universal PSB molecular marker.

Organic Phosphate Solubilization

Considering there are many different organophosphorus compounds, both natural and synthetic, we also find many different exoenzymes that can break these compounds down. Breaking organic P compounds down to either ionic phosphate or low molecular-weight organic phosphate is a step by step process that each requires specific enzymes from a selected group of microbes (Singh & Allan, 2006). A composting trial in 2018 was able to determine fifteen main organic P solubilizing bacteria which were significantly related to changes in the soluble P fraction (Wei, Zhao, Lu, Cao, & Wei, 2018).

Organic phosphates are proposed to be mineralized by extracellular enzymes and transmembrane enzymes of the P-solubilizing organism. The esterphosphate bonds in organic phosphates are hydrolyzed which releases P from the compound. These exoenzymes transform the high molecular weight P compounds to low molecular weight compounds. The majority of the exoenzymes originate from soil microbes, resulting in an increased concentration in the rhizosphere (Tarafdar, 1986).

Some examples of these enzymes are phosphatase, phytase, phosphonatase, and C-P lyase (Prabhu, Borkar, & Garg, 2018). The two most common enzymes are non-specific phosphohydrolases (also called phosphatases) and phytase. Phosphatase which

hydrolyzes phosphoric acid monoesters into P and a hydroxyl molecule (Zhu et al., 2018) Depending on the pH, bacteria are able to release acidic or alkaline specific phosphatases. These are non-specific and are the most abundant, they can mineralize the majority of organic P in the soil (Ragot, Kertesz, Meszaros, Frossard, & Bunemann, 2017). Phytase hydrolyzes phytic acid (inositol P) to produce phytate. By hydrolyzing the phosphomonoester bonds in phytate, phytase releases lower forms of myoinosital phosphates as well as inorganic phosphate (Zhu et al., 2018). 63% of cultured soil bacteria were able to grow on agar medium with phytate as the only carbon and phosphorus source, however in liquid medium with phytate as the only carbon and phosphorus source only 39-44% were able to grow (Richardson & Hadobas, 1997). The C-P bond of organophosphonate can be cleaved by phosphonatase and C-P lyase, and there are many other enzymes that can cleave the phosphoester bonds in nucleotides, and sugar phosphates (Wanner, 1996).

There are many factors that have been shown to influence the production and activity of the exoenzymes. Some of the factors include soil properties such as pH, organic matter, temperature, salinity, depth, organism interactions, and plant cover (Zhu et al., 2018). Phosphatase activity is impacted by soil pH, and most commonly the activity increases as soil pH increase, with the exception of acid phosphatase (Acosta-Martínez & Tabatabai, 2000). The addition of manure to a field increased the activity of the soil phosphatase, potentially caused by the indirect benefits of the manure such as an increase in pH and microbial activity. Organic matter also showed a strong correlation to phosphatase activity. Organic matter helps to protect and sustain

enzymes in their active forms. Forty soil samples were tested for organic carbon and enzyme activity and higher organic carbon soil led to higher phosphatase activities (Deng, 1997). Temperature plays a critical role for many of soil processes such as microbial metabolism, and nutrient cycling, and is correlated to an increase in phosphatase activity in the soil (Zhu et al., 2018). Soil depth is also a key factor, the lower soil depth, the lower the phosphatase activity. Deep tillage and soil structure also play a significant role in enzyme activity. Deep tilling increased the levels of microbial released exoenzymes. Most likely due to the fact that, in the short term, the deep tillage increased microbial activity (Ji et al., 2014). Clay particles tend to have a higher cation exchange capacity, high surface area, and smaller pores which hold capillary water and organic matter better. Clay soil had a 10.9% increase in phosphatase activity levels over the loam soil, which most likely is due to the increase in microbial activity producing more phosphatase enzymes (Ji et al., 2014). An increase in salinity and sodicity content decreased the phosphatase enzyme activity with an exponential trend and a linear trend, respectively (Rietz & Haynes, 2003). Another stressor of the exoenzyme activity is heavy metal concentrations. Heavy metals are able to modify protein configuration which inhibits enzyme synthesis. A study was conducted over a ten-year period to test how heavy metals such as Zn, Cu, Ni, V, and Cd affected microbial populations and enzyme activity. After ten years of incorporation of differing levels of heavy metals, phosphatase activity decreased (Kandeler et al., 2000). Considering phosphatase enzymes are produced and released by microbes, there are many factors in the soil that

can alter the activity of those exoenzymes and affect the amount of soil organic phosphate that are mineralized.

Genetics of Organic Phosphate Solubilization

The regulator mechanism that is most commonly involved in producing exoenzymes that solubilize insoluble organic phosphate is referred to as the Pho regulon. This was first characterized in *Escherichia coli* by Wanner and Chang (1987). The Pho regulon is the key to most all aspects of organophosphate metabolism. When the Pho regulon is activated due to low phosphate levels, approximately 50 different genes are expressed upstream of the phosphate-regulated gene activation (Lubin, Henry, Fiebig, Crosson, & Laub, 2016). When the organism senses low phosphate levels and the genes are expressed, 90 phosphate-starvation-inducible proteins were found to be activated (Wanner & Chang, 1987). The Pho regulon is responsible for activating important mechanisms such as exoenzymes that solubilize P, transporters that bring P into the cell, and enzymes needed for P storage (Wanner & Chang, 1987).

The exact mechanism that detects low P concentrations is unknow, however two of the component regulatory proteins of the Pho regulon have been studied in detail. These two proteins have been identified and specifically named in several different bacteria. They are named PhoR-PhoB in *E. coli* (Tommassen, de Geus, Lugtenberg, Hackett, & Reeves, 1982), PhoR-PhoP in *Bacillus subtilis* (Hulett et al., 1994), and PnpR-PnpS in *Streptococcus pneumoniae* (Novak, Cauwels, Charpentier, & Tuomanen, 1999), and many more. It is a two-component system that enable bacteria to transcribe the

needed genes during phosphate limitation. When referring to the *Bacillus* proteins, PhoP and PhoR, they are encoded by the *PhoRP* operon which is transcribed from two sigma factor dependent promoters, P_1 and P_2 (Pragai et al., 2004).

The sensing protein, PhoR, is an inner-membrane histidine kinase. This sensor kinase is self-phosphorylated under phosphate scarcity. The second protein, PhoB in *E. coli*, is a cytoplasmic transcriptional response regulator protein. The sensor protein PhoR then transfers the phosphorylation to the aspartic residue of the regulator protein PhoB. The regulatory protein binds to a specific DNA sequence, called the PHO box, to either activate or repress the Pho genes (Santos Beneit, 2015). The (*phoB)* gene is self-regulated and controlled by the level of available phosphate (Wanner & Chang, 1987). The most common enzymes that are released during a P starvation period by the Pho regulon to aid in the solubilization of the nearby reserves are; alkaline phosphatase (*phoA*), phospholipases (*phoD*), phytase (*phyC*), glycerophosphodiester (*glpQ*), 5'-nucleotidases (*ushA*). There are two different kinds of transporters activated by the Pho regulon, high specificity and low affinity transporters. The P-specific transporters (*pst*) are the most conserved members of the Pho regulon in all bacteria, and the low affinity Pi-transports (*pit*) are less conserved between bacterial species (Santos-Beneit, Rodriguez-Garcia, Franco-Dominguez, & Martin, 2008).

Pragai et al. (2004) showed that the sigma factor dependent promoters P_1 and P_2 are needed for phoPR operon transcription. PhoPR transcription was only detected from the P_1 promoter when the phosphate levels were adequate or when (*phoR)* was replaced with the null mutant. However, they found that during P starvation the phoPR

operon, as well as the P_1 and P_2 promoters were up regulated. The activation of the P_1 and P_2 promoters also seem to be influenced by the presence of other sigma factors (Pragai et al., 2004).

The (*ppk)* gene is also expressed by the activation of the Pho regulon. The (*ppk)* gene is involved in the storage of inorganic P (Ghorbel, et al., 2006). The (*ppk)* gene activates the reversible polymerization of the terminal phosphate of ATP to form a long-chain polyphosphate due to the expression of the enzyme catalyst polyphosphate kinase (PPK) (Masahiro, Elliott, & Arthur, 1992). In the presence of *Streptomuces lividans* TK24, and sufficient levels of phosphate the (*ppk)* gene expression was low. A repressor, that uses ATP as a corepressor, controls the expression of the (*ppk)* gene. When phosphate is limited the (*ppk)* gene is up regulated by the PhoPR operon (Ghorbel, et al., 2006). Ghorbel et al. (2006) suggests that PPK potentially acts as a nucleoside diphosphate kinase, based on the fact that a (*ppk)* mutant had low levels of polyphosphate as well as longer polyphosphates compared to the wild-type strain (Ghorbel, Kormanec, Artus, & Virolle, 2006).

The protein PhoU plays a role in the regulation of phosphate transport into the cell. PhoU has most commonly been linked to the homeostasis of cellular phosphate modulation by PstSCAB transporters (Hsieh & Wanner, 2010). You can typically find the *phoU* gene and the *pstSCAB* genes colocalized. The *phoU* gene encodes a protein approximately 25 KDa in size that controls the activation of the PhoR-PhoP/B phosphate limitation response (Willsky & Malamy, 1980). The *pstSCAB* genes encode for a high-affinity, ABC-type Pi transport system (Yuan, Zaheer, & Finan, 2006). The interaction of

these two protein groups have been inconclusive, until DiCenzo et al (2017) were able to delete the *phoU* gene, which proved very difficult in past studies. They showed a link between PhoU and PstSCAB P transported in *Sinorhizobium meliloti*. Past studies were not able to see as clear of a relationship because they mainly ran their test in a P deficient environment. However, when diCenzo et al. (2017) tested the *phoU* mutant in elevated phosphate media the PstSCAB-mediated P transport was not regulated properly and there was a toxic accumulation in the cell. In P starved media, PhoU increased the amount of Pi transported into the cell by PstSCAB, and when phosphate levels were elevated, PhoU rapidly responded by decreasing the PstSCAB transport rate.

Analytical measurement of phosphorus

There have been numerous studies to determine possible isolates and techniques for using phosphate solubilizing microbes (PSM) as biofertilizers in agriculture. Most commonly researchers have conducted studies to determine which microbes have the greatest phosphate solubilization ability, as well as provide other plant growth promoting benefits. However, there needs to be a greater emphasis on performing in-vivo studies with the potential biofertilizer and the appropriate plants.

There are two main ways of determining phosphate solubility, qualitative or quantitative. The most commonly used way is with a qualitative process using NBRIP (National Botanical Research Institute's phosphate growth medium), or Pikovskaya agar, and measure the clearing zone produced by a stab method. Phosphate solubilization ability is estimated by measuring the colony diameter and the clearing "halo" zone. The

equation that has been used to determine the phosphate solubilization index (PSI) is as the follows (Dipak Paul, 2017):

$$Phoshate\ solubilization\ index, PSI = \frac{Colony\ diameter + halozone\ diameter}{Colony\ diameter}$$

To determine the amount of insoluble P released by the organism, a quantitative test is needed. There are several ways of measuring the release of P quantitatively, some of the methods are as follows; Molybdenum-blue method (also called ascorbic acid method), Vogel method, Vanado-molybdate colorimetric method, and stannous chloride method. The molybdenum-blue method was initially determined for the measurement of phosphate in water samples (Murphy, 1962). In this method phosphate and molybdate form 12-molybdophosphoric acid in an acid solution which is subsequently reduced to phosphomolybdenum blue and can be measured at 882 nm (Nagul, McKelvie, Worsfold, & Kolev, 2015). The Vogel method uses the UV-vis spectrophotometer at 830 nm to measure the solubilized P measured with sodium molybdate and sulphuric acid which reacts and gives a blue colored complex. The Vanado-molybdate colorimetric method was published in the APHA Method 4500: Standard Methods for the Examination of Water and Wastewater document in 1992 (Greenberg, Clesceri, & Eaton, 1992). In this assay the orthophosphate reacts with ammonium molybdate under acidic conditions which forms a heteropoly acid, molybdophosphoric acid. A yellow color is formed by the formation of vanadomolybdophosphoric acid in the presence of vanadium. The yellow color is determined at 470 nm and is proportional to the level of phosphate in solution

(Technology, 2014). And finally, the Stannous Chloride method was published in the APHA Standard Methods, 22nd edition, Method 4500-P in 1999, and was also initially developed for the measurement of phosphorus in water (Nivens, Arora, Emery, Poff, & Schindler, 1999). In this method the orthophosphate reacts with ammonium molybdate which forms molybdophosphoric acid similar to the other tests, but then this is reduced by stannous chloride to the intensely colored molybdenum blue which can then be quantified colorimetrically (CHEMetrics, 2017).

However, there has been some discrepancies between the plate qualitative test and the broth quantitative test. In some instances when the solubilization index based on the clearing zones is low, the quantitative results are high, and vise-versa. Baig, Arshad, Zahir, and Cheema (2010) found that there was no correlation between the qualitative and quantitative phosphate solubilization results. In their research, some isolates that did not create clearing zones proved to be good P solubilizers when quantitatively measured. They suggest the quantitative method should only be concluded as reliable (Baig, Arshad, Zahir, & Cheema, 2010).

The use of PSB as biofertilizers

With the growing need for economical and environmentally safe alternatives to fertilizers used in the agriculture industry, the biofertilizer market is expanding. Grand View Research Inc. conducted a study on biofertilizers and estimated that the industry will reach USD 1.65 billion by the year 2022. This is driven by both environmental concerns as well as more rigorous government regulations of fertilizer utilization. They

found that the PSB biofertilizer market is expected to be the fastest growing portion of this industry, with a compound annual growth rate of 13.9% from 2015 to 2022 (Grand, 2018). There have been lots of greenhouse and field trials to study how PSB respond in a realistic environment. However, lots of testing is needed due to the vast diversity of bacterial species, and environmental conditions.

Valetti, Iriarte, and Fabra (2018) isolated bacteria from the rhizosphere of rapeseed plants, and out of the 40 bacterial isolates they found fourteen (37.8%) were capable of solubilizing phosphate. There was also an increase in rapeseed yield on the field trials from several isolates tested. The range of the percent yield increase was 21-44%, and several had a greater increase than commercial fertilizer application (Valetti, Iriarte, & Fabra, 2018).

Nassal et al. (2018) showed that *Pseudomonas* sp. RU47 (RU47) was able to increase plant performance of tomatoes. The increased plant performance was due to an increase in P uptake, an increase in microbial phytase activity in the rhizosphere, as well as a promotion of the indigenous *Pseudomonads* and phytohormones in the rhizosphere (Nassal et al., 2018). *Azotobacter salinestris* is not only a strong phosphate solubilizer, but is able to fix nitrogen, produce IAA, suppress fungal growth, and can surprisingly completely biodegrade pendimethalin, a herbicide (Chennappa, Sreenivasa, & Nagaraja, 2018).

Not all isolates are initially isolated from the plant or root zone. Three bacteria were isolated from the gut of an earthworm (*Metaphire posthuma)* which happened to

have plant growth promoting abilities. These three bacteria; *Bacillus licheniformis, B. megaterium, and Staphylococcus haemolyticus* were able to solubilize P even in the presence of heavy metals, and produce plant beneficial indole acetic acid (Biswas et al., 2018).

The emphasis on researching potential biofertilizer microbes has been increasing over the recent years, and the biodiversity of the bacteria that have P-solubilizing capabilities is quite high. Numerous species of *Bacillus* have been found to make P bioavailable (Banik & Dey, 1982, 1983; Gupta, Singal, Skankar, Kuhad, & Saxena, 1994; Sadiq, Jahangir, Nasir, Iqtidar, & Iqbal, 2013; Vazquez, Holguin, Puente, Lopez-Cortes, & Bashan, 2000), species of *Pseudomonas* (Bar-Yosef, Rogers, Wolfram, & Richman, 1999; Mahanta et al., 2014; Tani, Akita, Murase, & Kimbara, 2011), *Arthrobacter* species (Banik & Dey, 1982; Yi, Huang, & Ge, 2008), *Enterobacter* species (Hwangbo et al., 2003; Shahid, Hameed, Imran, Ali, & van Elsas, 2012; Thaller et al., 1995; Vazquez et al., 2000), *Kluyvera, Chryseomonas, Vibrio, Xanthobacter, Micrococcus, Klebsiella*, and more (Banik & Dey, 1982; Ohtake et al., 1996; Vazquez et al., 2000).

In conclusion, over the last century agriculture has been increasing its yields, most commonly by increasing the chemicals being applied. However, this has led to a plateau in crop production and a detrimental impact on the environment. Phosphate fertilizer is a finite resource and we need to find more economical ways of supplying P to plants. Bioinoculants are an environmentally friendly and economical way of increasing crop yields. Novel isolates are continuing to be discovered, and the there are numerous benefits from the PGPB. One of the fastest growing sectors of the

bioinoculant market are PSB. These bacteria have continued to prove beneficial in providing the plant with P and increasing yield.

We have characterized and named several potential bioinoculants for the agriculture market. These novel isolates have been identified in the Oxalobacteraceae family and have potential to be commercialized as bioinoculants. In addition, several isolates have been cultured and tested for their PSB activity. A few strong biofertilizers candidates were identified and have been researched and tested for P solubilizing abilities. These isolates prove to be promising alternatives to conventional farming practices.

CHAPTER 2

Draft genome sequence of *Pseudoherbaspirillum* gen. nov., sp. nov. OM1, a novel bacterium isolated from farm soil

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Running title: Pseudoherbaspirillum sperare

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Abstract

We sequenced a bacterial isolate designated strain OM1, which could potentially be a new member of the *Oxalobacteraceae* family that was isolated from actively farmed soil. Sequence analysis showed an assembled draft genome size of 4,709,175 bp, containing a predicted total of 4,967 protein-encoding genes, 2 *rRNAs* operons, and 44 *tRNAs*.

Announcement

The family *Oxalobacteraceae* is comprised of 13 distinct genera which are all Gram negative, non-spore forming, mostly mesophilic with only a small number of psychrophilic species (Baldani et al., 2014). Some genera are found in soils, can be closely associated with plants, and can colonize plants as endophytes (Pereira, do Amaral, Dall'Asta, Brod, & Arisi, 2014). *Oxalobacteraceae* also form close relationships with other soil microbes, such as arbuscular mycorrhizal fungi (Offre et al., 2008) and can promote spore germination, hyphal growth and root colonization (Scheublin, Sanders, Keel, & van der Meer, 2010). The genus *Herbaspirillum* contains species with plant growth promoting capabilities that

could serve as microbial fertilizers in agricultural applications, and increase the yield of economically important crops, such as rice and sorghum (Hoseinzade et al., 2016; Schlemper et al., 2018).

Pseudoherbaspirillum sperare (OM1) was collected on September 12th, 2016, from the subsurface of a sandy-loam soil near the Highland Township, Michigan, USA (44.1251, -85.2112). The cultures were isolated on R2A media at 30°C. Genomic DNA extraction was performed using the AllPrep Bacterial DNA/RNA/Protein kit (QIAGEN Inc., Germantown, MD) and the protocol for bacterial gDNA extraction. A genomic library was prepared using the Illumina Nextera platform (San Diego, CA), size selected to an average fragment length of 475 bp and sequenced by the Illumina NextSeq paired-end v2 Chemistry on v2.5 Flowcells at 150 bp per read with a 20x target coverage for reads.

We obtained 384,692 total reads with an average length of 148 bp (56,755,616 total bases). Genome assembly was carried out using SPADES version 3.11.0, which produced 524 contigs, with an N_{50} value of 11,660 bp (range: 2,500 - 51,540 bp) and a total assembled size of 4,709,175 bp. The G+C content was 63.81%. Reads that met quality control standards, were mapped to contigs with an average genome coverage of 20X. Assembly quality using BUSCO (Simao, Waterhouse, Ioannidis, Kriventseva, & Zdobnov, 2015) revealed a measured completeness (39 single copy BUSCO's, one duplicate BUSCO) of 97.5%. Contigs were annotated using the Galaxy platform(Afgan et al., 2018), PATRIC 3.5.28 (Wattam et al., 2017) and RAST 2.0 (Aziz et al., 2008), which identified in total 5,013 genes, 4,967 protein-coding genes, 2 rRNA operons, and 44 tRNA genes.

To determine taxonomic novelty, we used the MiGA (Rodriguez et al., 2018) platform with the RefSeq pipeline, and determined that OM1 belongs to the family *Oxalobacteraceae* with p-values of 0.265 and 0.581 on the genus and species level, respectively. Using the average amino acid identity between genomes, the highest similarity and genome relatedness was found to *Herbaspirillum hiltneri N3 (Guizelini et al., 2015)* at 65.13%. Using genome-to-genome-distance calculations through the website GGDC (Meier-Kolthoff et al., 2013), NCBI BLAST (Altschul, Gish, Miller, Myers, & Lipman, 1990) revealed five highly similar genomes after using the full 16S rRNA contig from the genome of OM1 as the search query. GGDC calculates intergenomic distances and DNA-DNA hybridization probability values with additional confidence intervals. When comparing the *P. sperare* strain OM1 genome to these 6 similar genomes (*Collimonas arenae* strain Cal35, *Herbaspirillum seropedicae* strain Z67, *Herbaspirillum hiltneri* N3, *Herbaspirillum seropedicae* strain AU14040, *Massilia sp.* WG5, *Herbaspirillum sp.* Meg3), all DDH probability values were 0 % (less than the 70% scientific community threshold) (Tindall, Rossello-Mora, Busse, Ludwig, & Kampfer, 2010; Wayne et al., 1987), suggests that strain OM1 is a novel species within a new genus. Using the Type Strain Genome Server to screen similar type strains using the 16S rRNA gene of strain OM1 and related species, OM1 was found be similar to *Noviherbaspirillum denitrificans* (AB542397) (Meier-Kolthoff & Goker, 2019). However, bootstrap values suggest OM1 forms its own unique branch.

Data availability

The genome sequence of OM1 has been deposited at DDBJ/EMBL/GenBank

(https://www.ncbi.nlm.nih.gov/bioproject/PRJNA529104) under the BioProject number: PRJNA529104, BioSample number: SAMN11257890 and accession number: SPQI00000000.

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Pseudoherbaspirillum sperare gen. nov. sp. nov., a novel species and genus of the Oxalobacteraceae

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Keywords: Oxalobacteraceae,

Abbreviations: ML, maximum-likelihood, AAI, average amino acid index, DSMZ, German Collection of Microorganisms and Cell Cultures GmbH

The GenBank/EMBL/DDBJ accession numbers for the genome of *Pseudoherbaspirillum sperare gen. nov. sp. nov* strain OM1 are listed under the BioProject number: PRJNA529104 (BioSample number: SAMN11257890 and Accession number: SPQI00000000)

Status: close to submission to the journal International *Journal of Systematic and Evolutionary Microbiology*. Papers are ready and waiting on paperwork for the culture collection banks.

Abstract

A novel member of the family *Oxalobacteraceae* was isolated from farmed soil in North Carolina, was designated strain OM1. Based on 16S rRNA phylogenetic analysis and full genome analysis, it was discovered that the bacterial isolate did not belong to any known genus under the *Oxalobacteraceae* family. The closest similar family members based on 16S rRNA are: *Noviherbaspirillum denitrificans* strain TSA40, *Noviherbaspirillum agri* strain K-1-15 and *Noviherbaspirillum autotrophicum* strain TSA66 (98.51, 97.81 and 97.57 percent identity respectively). Biochemical testing performed with Biolog Gen III microplates, showed that OM1 has similar biothermal characteristics with *Janthinobacterium agarcidamnosum* strain W1r3. Average amino acid identity genome comparison revealed a similar member, *Herbaspirillum sp*. meg3 NZ to OM1 at an index percentage of 65.2% and a 0% DNA-DNA hybridization greater than or equal to 70. Using

the results from biochemical, phylogenetic, genome and physiologic analysis, OM1 is considered a novel genus of the family of *Oxalobacteraceae* and the name of *Pseudoherbaspirillum sperare gen. nov. sp. nov* strain OM1 is proposed.

Introduction

The family of *Oxalobacteraceae* is phylogenetically related to the order Burkholderiales, is a subclass of Betaproteobacteria and is comprised of 13 distinct genera: *Collimonas*, *Duganella*, *Glaciimonas*, *Herbaspirillum*, *Herminiimonas*, *Janthinobacterium*, *Massilia*, *Noviherbaspirillum*, *Oxalicibacterium*, *Oxalobacter*, *Pseudoduganella*, *Telluria* and *Undibacterium*. The bacteria within this group are all Gram negative, non-spore forming, and generally mesophilic with the exception of a small number of psychrophilic species (Jose Ivo Baldani et al., 2014). *Oxalobacteraceae* are present in a wide range of habitats, such as air, freshwater, soils, Antarctic soil, plant roots, rhizosphere and phyllosphere (Ofek, Hadar, & Minz, 2012). Some species/strains of the family are plant associated, and can be mild plant pathogens, while others are endophytic, and have plant growth promoting capabilities (Tomás Pellizzaro Pereira, do Amaral, Dall'Asta, Brod, & Arisi, 2014). These plant beneficial capabilities can for example include the suppression of plant pathogens (Toumatia et al., 2016) nutritional benefits (Santos et al., 2014) growth promotion via the production of plant growth hormones (Cerboneschi et al., 2016). The root associated or endophytic genus *Herbaspirillum*, for example, has been used as microbial inoculant in agricultural applications, and has been shown to increase the yields of economically important crops, such as sorghum (Schlemper et al., 2018).

In this article, we describe the novel strain OM1, which belongs to a new genus within the *Oxalobacteraceae* family. *P. sperare* was isolated from farmland and is most closely related to the genus *Herbaspirillum* but differs phenotypically and genetically from known *Herbaspirillum* strains. We propose to name this isolate, *Pseudoherbaspirillum sperare gen. nov. sp. nov,* strain OM1.

Isolation and ecology

Pseudoherbaspirillum sperare, strain OM1 was isolated from the subsurface of a sandy-loam farm soil near the Highland Township (Michigan, USA; 44.1251 N, 85.2112 W). The mean summer temperature of the area ranges from 18-20°C, while mean annual precipitation can be 600 to 813 mm. According to a soil profile from the Web Soil Survey website (Soil Survey Staff, 2017), the soil pH of the area was 6.1, the soil is well drained and primarily used as farmland and has a low salinization risk (Soil Survey Staff, 2017). The soil sample was treated with a phosphate saline buffer solution, and samples of the slurry were streaked on R2A media plates and cultured at 30°C (Reasoner & Geldreich, 1985). After repeated single cell colony isolation, pure colonies were transferred to a 20% glycerol solution and stored at -80°C for future analysis.

Phenotypic characterization

The cells of *P. sperare* strain OM1 are Gram negative rods with a chain like cellular arrangement. In culture on R2A media, colonies appeared to be white-cream, in circular form, with a slightly raised elevation and an entire margin at the edge of the colony.

For the chemotaxonomic characterization, we compared *P. sperare* to 16 reference strains from the *Oxalobacteraceae* family (Table 5). The reference strains were selected based on a 16S rRNA BLAST search and were obtained from three different culture collections: American Type Culture Collection (ATCC, Manassas, USA), Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures (DMSZ, Braunscheig, Germany) and VTT Technical Research Centre of Finland (Espoo, Finland). All strains were chemotaxonomically characterized by the Microlog system (Biolog, Hayward, USA) using Gen III 96 well plates that contain 94 phenotypic tests that are divided into 71 metabolic substrate tests and 23 chemical sensitivity tests (Bochner, 1989).

Table 5. Bacterial isolates purchased from culture collection banks and their corresponding catalog codes.

Bacterial Isolate	Culture Collection Catalog Code	Culture Collection
Massilia namucuonensis	2159	DSMZ
Massilia umbonata	26121	DSMZ
Massilia dura	17513	DSMZ
Massilia aerilata	19289	DSMZ
Duganella zoogloeoides	25935	ATCC
Massilia albidiflava	17472	DSMZ
Noviherbaspirillum aurantiacum	TSD-69	DSMZ
Undibacterium terreum	102222	DSMZ
Oxalobacter formigenes	4420	ATCC
Oxalicibacterium faecigallinarum	21641	DSMZ
Pseudoduganella violaceinigra	15887	DSMZ
Janthinobacterium agaricidamnosum	9628	DSMZ
Collimonas arenae	21398	DSMZ
Glaciimonas singularis	100199	DSMZ
Herbaspirillum seropedicae	6446	DSMZ
Herminiimonas contaminans	28178	DSMZ
Telluria mixta	ATCC 49108	ATCC
Naxibacter alkalitolerans	VTT:CAS29	VTT

For the test, strains were briefly grown on the required media and temperature according to the recommendations of the culture collection (Table 5), and then transferred into inoculation fluid C at room temperature (Biolog, Hayward, USA) according to the instruction of the manufacturer. Then 100 µL of the culture, with an absorbance of 98%, were aliquoted into each well of the plate and then incubated at 30°C. After 2-5 days, depending on the growth rate of the different strains, the plates were read and the results were entered into the Microlog program as "+" for a positive color change, "-" for a negative, and "+/-" for a borderline result. The Microlog results were then converted from +, -, -/+ into the numerical form 2,0,1, respectively. The results were then entered into R-studio(Ross Ihaka & Robert Gentleman, 1996) to create a non-metric multidimensional scaling plot to examine the clustering of the different bacterial isolates (Figure. 5). OM1 clustered closely to *Janthinobacterium agaricidamnosum* strain W1r3 indicating the phenotypic similarities between the two isolates. However, when the genomes of *Janthinobacterium agaricidamnosum* strain W1r3 and OM1 are compared, they are not genetically similar, but could have a common ancestor.

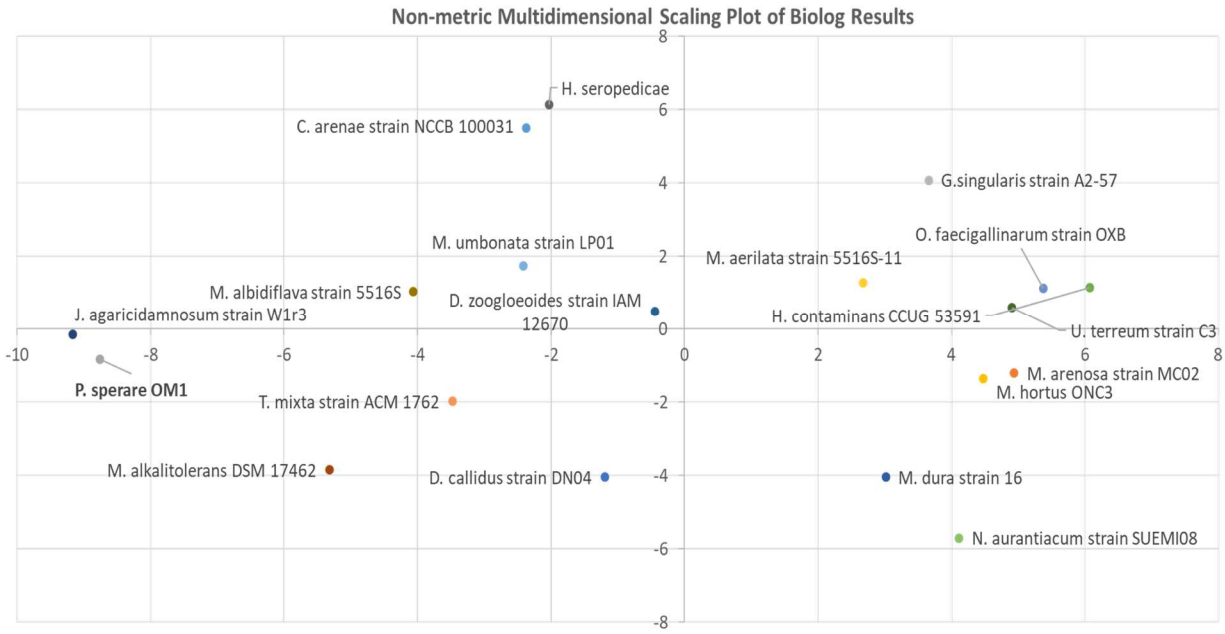

Figure 5. Non-metric multidimensional plot of OM1and similar Oxalobacteraceae family members' microlog results.

MALDI-TOF MS

Matrix-assisted laser desorption/ionization time of flight mass spectroscopy (MALDI-TOF MS) was performed by Novozymes A/S. A MALDI-TOF target plate was prepared by mixing bacterial cell samples and matrix solution and dried to trap the samples in place. The sample matrix is then ionized with a laser beam which accelerates the ions at fixed speed or flight. For microbial analysis, time of flight is used to measure the mass-to-charge ratio (m/z) of ions by determining the length of time an ion takes to go through the flight tube of the mass spectrometer. A spectrum is made that displays the peptide mass fingerprint of the bacterial isolate (Holland et al., 1996; Singhal et al., 2015). According to the MALDI-TOF analysis, OM1 forms a separate branch with *Massilia arenosa* MC02, a newly described species within the *Massilia* species, but differs from the other reference strains.

DNA isolation, sequencing and genome annotation

Genomic DNA extraction was performed using the AllPrep Bacterial DNA/RNA/Protein kit (QIAGEN Inc., Germantown, MD) and the protocol for gDNA extraction from bacterial colonies. A genomic library was prepared using the Illumina Nextera platform (San Diego, CA), and sequenced by the Illumina NextSeq paired-end v2 Chemistry on v2.5 Flowcells with 150 bp per read, a fragment length of 475 bp, and a 20X target coverage for reads.

We obtained 384,692 total reads with an average length of 148 bp (56,755,616 total bases). The genome assembly was carried out using SPAdes 3.11.0 (Nurk et al., 2013), which produced 524 contigs, with an N_{50} value of 11,660 bp and a total assembled size of 4,709,175 bp. The G+C content was 63.81%. Reads that met quality control standards, were mapped to contigs with an average genome coverage of 20X. Assembly quality was assessed using BUSCO (Simao et al., 2015). The measured completeness (39 single copy BUSCO's, one duplicate BUSCO) was 97.5%. Initial annotation was performed using the PATRIC platform 3.5.28 (Wattam et al., 2017). Additional annotation was implemented using the Galaxy platform (Wattam et al., 2017) and RAST 2.0 (Aziz et al., 2008) and the NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et al., 2016), which identified in total 5,013 genes, 4,967 protein-coding genes, 2 rRNA operons, and 44 tRNA genes.

16S rRNA phylogeny

After obtaining the partial 16S rRNA sequence, NCBI BLAST was used to select the top 15 most similar 16S rRNA sequences. The sequences were downloaded as FASTA files and imported into MEGA 7 (Kumar, Stecher, & Tamura, 2016) and aligned by MUSCLE (Edgar, 2004). A best-fit DNA model, using the aligned sequences was created, and used to perform the maximum-likelihood phylogenetic analysis. The Kimura-2-gamma with invariant sites model (K2+G+I) (Jayaswal, Robinson, & Jermiin, 2007; Kimura, 1980) 1000 bootstrap replicates, and a partial deletion with 95% coverage cutoff was used to create the final maximum-likelihood tree (Figure 6) and to visualize it in MEGA7 and iTOL (Letunic & Bork, 2016). *Escherichia coli DSM 30083* was used to root the phylogenetic tree. According to the phylogenetic tree based on 16S sequencing, OM1 shares a common ancestor with *Noviherbaspirillum denitrificans* strain TSA40.

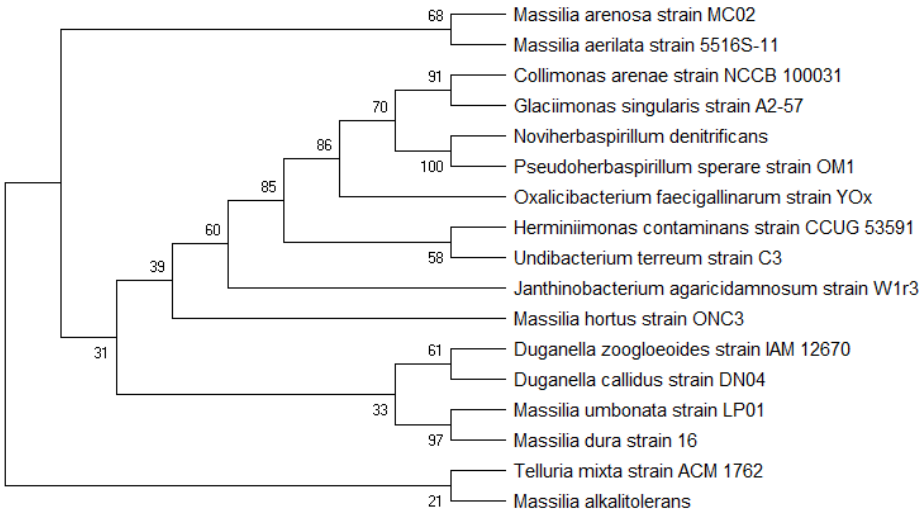

Figure 6. Maximum-likelihood phylogenetic tree based on the top similar 16S rRNA sequences from a BLAST search compared to OM1. Values at branch points indicate bootstrap support as percentages based on 1000 resampling (only values greater than 50% are shown).

Genome features

We used the MiGA (M. et al., 2018) platform and its prokaryotic pipeline to compare the *P. sperare* genome to other available genomes and found that *P. sperare* belongs to the family *Oxalobacteraceae,* but has low probability of being affiliated to any of the existing genera of this family. Using the average amino acid identity between genomes, the genome of *P. sperare* is most closely related to *Herbaspirillum sp. Meg3* with a similarity of 65.13% (Table 6). This low AAI is just above 65%, the threshold that MiGA typically applies to predict a new genus.

Table 6. Average amino acid index values of genomes similar to OM1 when compared to each other.

Similar Genomes to 034Z9T	**AAI (%)**
Herbaspirillum sp. meg3 NZ CP022736	65.2
Herbaspirillum hiltneri N3 NZ CP011409	65.13
Herbaspirillum rubrisubalbicans M1 NZ CP013737	64.56
Herbaspirillum seropedicae NZ CP011930	64.55
Herminiimonas arsenitoxidans NZ LT671418	64.3
Collimonas fungivorans Ter331 NC 015856	63.91
Janthinobacterium sp. LM6 NZ CP019510	62.2
Massilia sp. WG5 NZ CP012640	61.78
Oxalobacter formigenes NZ CP018787	57.77
Cupriavidus taiwanensis NZ LT984801	56.55

Using the full 16S rRNA contig from the genome of *P. sperare* as a the search query and genome-distance calculations through the website GGDC (Meier-Kolthoff et al., 2013), five highly similar genomes were obtained from NCBI BLAST (Altschul S.F., 1990). By comparison to similar phylogenetic neighbors, GGDC calculates intergenomic

distances and DNA-DNA hybridization (DDH) probabilities through the digital analysis of 3 different models. When the genome of *P. sperare* genome was compared to the 5 most similar genomes, the DDH probability values were lower than 70% for all prokaryotic species delineations (Table 7). A threshold of <70% DDH similarity is typically applied to justify the establishment of a new species (Alexander F. Auch, von Jan, Klenk, & Göker, 2010). Based on these data, we propose that OM1 represents a novel species of a novel genus within the *Oxalobacteraceae* family and should be taxonomically referred to as *Pseudoherbaspirillum sperare* strain OM1.

Table 7. GGDC with OM1 as the reference genome and was compared to 6 similar genomes based on the largest contig in the assembly.

	Formula 1				
Query genome	Reference genome	DDH	Model C.I.	Distance	Prob. DDH >= 70%
OM1_genome	Collimonas_arenae_strain_Cal35	13.5	[10.7 - 16.8%]	0.9478	0
OM1_genome	Herbaspirillum_seropedicae_strain_Z67	14.7	[11.9 - 18.1%]	0.8902	0
OM1_genome	Herbaspirillum_hiltneri_N3	15.1	[12.2 - 18.6%]	0.8734	0
OM1_genome	Herbaspirillum_seropedicae_strain_AU14040	14.7	[11.8 - 18.1%]	0.8916	0
OM1_genome	Massilia_sp._WG5	15.6	[12.6 - 19%]	0.8565	0
OM1_genome	Herbaspirillum sp. Meg3	13.7	[10.9 - 17.1%]	0.9361	0
OM1_genome	Formula 2				
OM1_genome	Reference genome	DDH	Model C.I.	Distance	Prob. DDH >= 70%
OM1_genome	Collimonas_arenae_strain_Cal35	20.4	[18.2 - 22.8%]	0.215	0
OM1_genome	Herbaspirillum_seropedicae_strain_Z67	22.2	[19.9 - 24.6%]	0.1979	0
OM1_genome	Herbaspirillum_hiltneri_N3	21.5	[19.3 - 24%]	0.204	0
OM1_genome	Herbaspirillum_seropedicae_strain_AU14040	22.2	[19.9 - 24.6%]	0.1975	0
OM1_genome	Massilia_sp._WG5	21.2	[19 - 23.7%]	0.2066	0
OM1_genome	Herbaspirillum sp. Meg3	21.1	[18.8 - 23.5%]	0.2084	0
OM1_genome	Formula 3				
OM1_genome	Reference genome	DDH	Model C.I.	Distance	Prob. DDH >= 70%
OM1_genome	Collimonas_arenae_strain_Cal35	13.8	[11.4 - 16.6%]	0.959	0
OM1_genome	Herbaspirillum_seropedicae_strain_Z67	14.9	[12.5 - 17.8%]	0.9119	0
OM1_genome	Herbaspirillum_hiltneri_N3	15.3	[12.8 - 18.2%]	0.8992	0
OM1_genome	Herbaspirillum_seropedicae_strain_AU14040	14.9	[12.4 - 17.8%]	0.913	0
OM1_genome	Massilia_sp._WG5	15.6	[13.1 - 18.5%]	0.8861	0
OM1_genome	Herbaspirillum sp. Meg3	14	[11.6 - 16.8%]	0.9494	0

The *P. sperare* genome contains putative genes that could be indicative for a growth promotion effect on plants. We identified for example the siderophore gene, *brfD*, which has also been found in *Noviherbaspirillum humi* strain U15 and encodes the protein TonB that interacts with outer membrane receptors (OMR) and aids in the uptake of the ferric-siderophore complexes (Sundararaman, Srinivasan, & Lee, 2016; B. R. Wilson, Bogdan, Miyazawa, Hashimoto, & Tsuji, 2016). Genes for phosphate solubilization were also found, including: *phoU* and *ppK2*. *phoU* is part of the Pho regulon that regulates phosphate management in bacterial cells. This protein is a metal binding protein and controls autokinase of other Pho regulon genes (Santos Beneit, 2015). *ppK2* codes for the polyphosphate kinase 2, which can utilize polyphosphates to produce GTP and was also found in Duganella (Ishige, Zhang, & Kornberg, 2002).

Proposal Pseudoherbaspirillum sperare gen. nov. sp. nov, strain OM1

P. sperare strain OM1 can be differentiated from other type strains and members of the *Oxalobacteraceae* family by the Microlog phenotyping system (Table 5), phylogenetic analysis and whole genome analysis. Microlog testing and comparison showed that the isolate was most similar to *Janthinobacterium,* however, the colony morphology and color of *P. sperare* are quite different than that of the *Janthinobacterium* isolate that was tested. It is not genetically similar in a significant fashion. The initial growth media with which these isolates were prepared (i.e. R2A agar or nutrient agar, ect.) also vary between each isolate and help to differentiate nutrient requirements for each isolate. AAI values and DDH values provided by MiGA and GGDC, respectively, had values lower than 70% cutoff (Tables 5 & 6), which suggests that isolate *Candidatus*

Pseudoherbaspirillum sperare gen. nov. sp. nov, strain OM1 is novel genus member of the *Oxalobacteraceae* family.

Description of Pseudoherbaspirillum sperare gen. nov. sp. nov

P. sperare strain OM1 was isolated from an agriculturally used soil close to the Highland Township (Michigan, USA; 44.1251, -85.2112). Cells are Gram-negative rods in chains. Colonies that are grown on R2A media are round shaped with a white-cream coloring and smooth and slightly raised surfaces. Growth occurs at 21-30°C. Cells can adapt to 1% and 4% salt solutions and various salt solutions according to the Microlog analysis (Table 5). Cells can also ferment various carbon substrates, assimilate various amino acid sources, which seems to be traits shared across the *Oxalobacteraceae* family. The genome of this isolate is 4.71 Mbp and has a G+C content of 63.81%.

Culture Collection Deposit

Strain OM1 was deposited into the American Type Culture Collection Depository and the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ).

Author Statements

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Ethical statement

The authors declare that the publication is not in consideration for any other journal, and that all authors agreed with its publication, and that no animal or human studies were carried out for this study.

Conflicts of interest

The authors declare that there are no conflicts of interest.

CHAPTER 3

Draft genome sequence of *Massilia* sp. MC02 isolated from a sandy-loam maize soil

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Abstract

From farmed corn soil in California, we isolated and sequenced a new member of the Genus *Massilia*: *Massilia* sp. MC02. *Massilia sp. MC02* has an assembled draft genome of 5,023,356 bp, with a total of 4,790 protein-encoding genes and 3,028 predicted proteins, 47 tRNA genes and 2 rRNA operons.

Announcement

The first species within the Genus *Massilia* was isolated from clinical samples (Scola, Birtles, Mallet, & Raoult, 1998). Since then, *Massilia* has been isolated from plant tissues (Chimwamurombe, Grönemeyer, & Reinhold-Hurek, 2016), water (Lu, Tian, Yu, Yang, & Zhang, 2018), air (Orthova, Kampfer, Glaeser, Kaden, & Busse, 2015), ice cores (B. X. Guo et al., 2016), and soils (Ren et al., 2018). *Massilia* has been shown to be abundant in the plant rhizosphere and to colonize roots (X. Li, Rui, Mao, Yannarell, & Mackie, 2014; Maya, Yitzhak, & Dror, 2012). Some species have plant growth-promoting capabilities such as the production of indole-3-acetic acid (Kuffner et al., 2010) or siderophores, and are involved in soil carbon and nitrogen cycling (Hrynkiewicz, Baum, & Leinweber, 2010).

Massilia is the most species-rich genus of the O*xalobacteraceae* and consists mainly of Gram negative, aerobic, nonspore-forming, motile rods.

Massilia sp. MC02 was isolated from a maize rhizosphere sample from a sandy loam soil in California on May 22, 2015. The geographical coordinates are 37.6058, -120.7478. Soil was added to phosphate-buffered saline and dilutions were plated on R2A plates, and incubated at 30°C for 1-2 days, followed by 20°C for 1-2 days. Sequential colony streaks were performed on R2A to acquire pure colonies. Genomic DNA was extracted from a freshly grown R2A broth culture using the AllPrep Bacterial DNA/RNA/Protein kit (QIAGEN Inc., Germantown, MD) following the kit protocol. The genomic library was prepared with Illumina Nextera (San Diego, CA), size selected to an average fragment length of 475 bp and sequenced using Illumina NextSeq paired-end v2 Chemistry on v2.5 flowcells at 150 bp per read. A target coverage of 20X was used, and the genome was assembled using SPAdes 3.11.0 (Bankevich et al., 2012). Default parameters were used for all software unless otherwise specified. We obtained 1,566,408 total reads, with an average read length of 148 bp. The total read length was 231,096,882 bp, with 275 contigs and a N50 value of 28,267 (range: 1,074 - 119,695 bp) and an L50 value of 49. The genome length was 5,023,356 bp with a GC content of 66.2%. Assembly quality using BUSCO (12) revealed a measured completeness of 95%. Gene prediction and annotation using PATRIC 3.5.27 (Wattam et al., 2017) resulted in a total of 4,790 protein coding sequences consisting of 1,762 hypothetical proteins and 3,028 proteins with functional assignments, 47 tRNA genes and 2 rRNA operons. MiGA (M. et al., 2018) revealed the closest related strain from the NCBI database is *Massilia armeniaca* NZ

CP028324 with an amino acid identity (AAI) of 67.37%. Based on the MiGA results, MC02 belongs to the *Massilia* genus, and was designated as *Massilia* sp. MC02.

Using Galaxy(Afgan et al., 2018), several genes with putative plant growth promoting characteristics were identified, such as a nitrate reductase gene (*napA*), several phosphatase genes (*ppk, phoA, phoB, phoD, phoR*), and biotin biosynthesis genes (*BioA, BioB, BioD, BioF*). Using RAST 2.0 (Overbeek et al., 2005), we identified 48 putative virulence genes, including 34 genes indicating a resistance to antibiotics and toxic compounds, 14 genes putatively involved in invasion and intracellular resistance, and 26 genes responsible for flagellar motility. Invasion and flagella genes are essential for attaching and entering plant cells (17), suggesting that MC02 is a plant endophyte.

Data availability

The complete genome sequence has been deposited in NCBI/EBI/GenBank under BioProject number PRJNA529270 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA529270/), BioSample number SAMN11263498 (https://www.ebi.ac.uk/biosamples/samples/SAMN11263498), accession number SPVF00000000, and SRA accession number SRX6098478 (https://www.ncbi.nlm.nih.gov/sra/SRX6098478[accn]).

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Massilia arenosa sp. nov., a novel addition to the *Massilia* genus, isolated from the soil of a cultivated maize field

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Keywords: *Massilia, Oxalobacteraceae*
Abbreviations:
Repositories: The genome has been deposited at DDBJ/EMBL/GenBank under the BioProject number PRJNA529270 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA529270), BioSample number SAMN11263498, and accession number SPVF00000000.

Status: close to submission to the journal International *Journal of Systematic and Evolutionary Microbiology*. Papers are ready and waiting on paperwork for the culture collection banks.

Abstract

A gram negative, rod-shaped bacterium, designated strain MC02, was isolated from a soil sample collected from California, USA. Phylogenetic analysis based on 16S rRNA and full genome revealed MC02 grouped within the *Massilia* genus. The closest *Massilia* species based on the 16S rRNA had a 96.79 percent identity. Based on biochemical testing using Biolog GenIII, MC02 is unique in several carbon and amino acid utilization parameters such as; D-Turanose, Inosine, and L-Serine. The most similar isolate in the MALDI-TOF database to MC02 was *Massilia aerilata* DSM19289 with a match score of 1.23 which is much below the 1.7 threshold for a novel isolate. Based on the full genome comparison the closest relative to MC02 based on average amino acid identity (AAI) had a 67.37% similarity, an OrthoANI similarity value of 77.14%, and a 0% probability of having a DNA-DNA-hybridization ≥ 70%, indicating MC02 is a novel species. MC02 has several beneficial genes such as; nitrate reductase, several phosphatases, and biotin biosynthesis genes. As well as, putative endophyte genes

comprising of 26 flagellar motility and 14 invasion and intracellular resistance genes. Based on biochemical, physiological, and genomic relatedness MC02 appears to be a novel species of the *Massilia* genus, and the name *Massilia arenosa* sp. nov. MC02 is proposed.

Introduction

Microorganisms in the plant rhizosphere play an enormous role for plant health. According to estimates there are up to 10^{11} bacteria per gram of rhizosphere soil (Egamberdieva et al., 2008). The number of newly identified microbial species from the rhizosphere is continuously increasing, particularly due to the development of novel culture-independent identification methods such as high-throughput sequencing techniques which have expanded the field of microbial ecology and revealed the vast microbial diversity (E. J. Stewart, 2012). Through these technologies, it is now clear that plants are able to shape the microbial community in their rhizosphere (Antoniou, Tsolakidou, Stringlis, & Pantelides, 2017; Berendsen, Pieterse, & Bakker, 2012), and that successional changes in the plant rhizosphere are characterized by decreases in both, taxonomic and phylogenetic diversity relative to bulk soil microbial communities (S. J. Shi et al., 2015).

The family *Oxalobacteraceae* is classified as member of the order *Burkholderiales*, the class *Betaproteobacteria*, and the *Proteobacteria* phylum (Garrity, Bell, & Lilburn, 2015). The family consists of Gram-negative bacteria that are most commonly nonspore-forming, mesophilic species (Scola et al., 1998). Since the incorporation of the *Massilia*

genus, many additional species have been added, and with its 41 named and described species, *Massilia* is the most species-rich genus of the *Oxalobacteraceae* (Euzeby, 1997). Since 2005, the *Massilia* genus now also includes the species of the *Naxibacter* genus that were first added to the *Oxalobacteraceae* family (Xu et al., 2005), but later reclassified based on 16S rRNA sequence and chemotaxonomic data (Kampfer, Lodders, Martin, & Falsen, 2011). The *Massilia* genus is most closely related to the *Telluria* and *Duganella* genera (B La Scola, Birtles, Mallet, & Raoult, 2000).

The first *Massilia* species was isolated from the blood of an immunocompromised patient in 1998 (Scola et al., 1998). *Massilia* is a niche divergent family of bacteria, and since their first discovery in 1998, species of the *Massilia* genus have been isolated from marama bean and poplar tissue samples (Chimwamurombe et al., 2016; Ulrich, Ulrich, & Ewald, 2008), water (Gallego, Sanchez-Porro, Garcia, & Ventosa, 2006; Lu et al., 2018), air (Orthova et al., 2015), and ice cores (B. X. Guo et al., 2016). *Massilia* organisms are most commonly isolated from soil samples and have been found to colonize root surfaces and are relatively abundant in the rhizosphere (Dohrmann & Tebbe, 2005; Maya et al., 2012). Culture-independent tools showed that *Massilia* bacteria can colonize the seed coat, radicle, roots, and even the hyphae of *Pythium aphanidermatum*, a phytopathogenic Oomycete that infects seeds, juvenile tissue, lower stems, and roots (Maya et al., 2012). *Massilia* are characterized as Gram-negative, rod shaped, aerobic, flagellated, non-spore forming bacteria. However, the species within the family are very metabolically and ecologically diverse (Jose Ivo Baldani et al., 2014).

There is an increasing interest in the *Oxalobacteraceae* family, and in particular in the *Massilia* genus, since the genus includes species with plant growth promoting characteristics from the plant rhizosphere and phyllosphere.

Massilia species have bioaugmentation abilities through their ability to grow in oil contaminated soils, and to contribute to oil removal (N. Ali et al., 2016). A strain of *Massilia* isolated from the rhizosphere of *Alyssum murale* was relatively resistant against high heavy metal concentrations of arsenic (20 mM), chromium (2.5 mM), nickel (15 mM) and zinc (10 mM) (Abou-Shanab, van Berkum, & Angle, 2007). The genus *Massilia* has disease suppressive abilities towards *Rhizoctonia solani* (Giuliano et al., 2018). *R. solani* is a plant pathogenic fungus with a wide crop host range, and the abundance of *Massilia* genera in organic soils was strongly linked to the observed disease suppression (Giuliano et al., 2018). Other strains of *Massilia* produce violacein and deoxyviolacein, broad-spectrum antibiotics, when the required amino acids L-tryptophan and L-histidine are available (Agematu, Suzuki, & Tsuya, 2011a). Several *Massilia* species produce siderophores and are able to hydrolyze gelatin and tributyrin, and other *Massilia* strains have cellulolytic and amylolytic activities (Hrynkiewicz et al., 2010).

Isolation

Massilia arenosa sp. nov. MC02, was isolated from a soil sample which was collected from a maize field in California on May 22, 2015. The geographical coordinates are 37.6058, -120.7478. The field was classified as Hanford sandy loam, and earthy peat soil. The field was furrow irrigated and the corn growth was stunted due to wet

conditions. The soil sample was suspended in 1X phosphate-buffered saline, and aliquots were plated on R2A medium and incubated first for 1-2 days at 30°C, and then for 1-2 days at 20°C. After repeated single cell colony isolation, pure colonies were transferred to R2A broth and grown at 30°C for 24 h, and then transferred to a 20% glycerol solution for storage at 80°C for future testing.

Morphology

Colony and cell morphology were determined by streaking the isolates onto R2A medium and growing them at 30°C for 3 days. Individual colonies were used to determine the colony morphology such as form, elevation, and margin, and to perform a Gram stain (Willgohs & Bleakley, 2009). To compare the colony morphology, we selected *Massilia aerilata* 5516S-11 (Leibniz-Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, DSMZ 19289) and *M. dura* 16 (DSMZ 17513) the two most similar species based on the BLAST results of the 16S rRNA sequence of MC02, that were publicly available through culture collections. *Massilia arenosa* colonies have a circular form, the elevation is flat, and the margins are entire, they are pin-point to medium in size and shiny, cream, and opaque in appearance (Figure 7A). The colony morphology of MC02 differed from both reference strains. *M. aerilata* formed larger yellow colonies with round and convex elevation (Figure 7B) (Weon et al., 2008), while *M. dura* formed circular, entire, convex, hard and compact, pale yellow colonies (Figure 7C) (Y. Q. Zhang et al., 2006). MC02 are Gram-negative rods, that are arranged as single cells or cell pairs (Figure 8).

Figure 7. Colony morphology of *Massilia arenosa* MC02 (a), *Massilia aerilata* 5516S-11 (b), *Massilia dura* 16 (c) after growth on R2A media grown at 30 °C for 3 days.

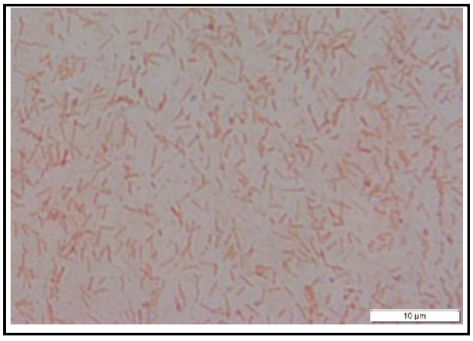

Figure 8. Gram-stain of *Massilia arenosa* MC02. Scale is 10 µm.

16S RNA phylogeny

Genomic DNA was extracted from a 1 ml suspension of a freshly grown R2A culture using the AllPrep Bacterial DNA/RNA/Protein kit (QIAGEN Inc., Germantown, MD) according to the kit protocol. The genomic library was prepared with Illumina Nextera (San Diego, CA) with a target insert size of 475 bp and sequenced using Illumina NextSeq paired-end v2 Chemistry on v2.5 flowcells at 150 bp per read. A target coverage of 20X was used, and the genome was assembled using SPAdes 3.11.0 (Bankevich et al., 2012). Default parameters were used for all software packages unless otherwise specified. Once sequenced, the 16S rRNA contig was used to determine the closest related sequences from the NCBI Basic Local Alignment Search Tool nucleotide database (Zhang Z., 2000). Sequences were downloaded as FASTA files and used to create a phylogenetic tree. We

used the default settings of the MUSCLE alignment option (Edgar, 2004) of MEGA 7 (Kumar et al., 2016) to align *M. arenosa* with the thirteen closest related sequences in the data base. *Burkholderia metallica* R-16017 was used to root the phylogenetic tree. The phylogenetic tree was created by using the maximum likelihood estimator with 1000 bootstrap replications, and partial deletion with a 95% coverage cut-off. Based on the phylogenetic tree, *M. arenosa* MC02 is most closely related to *Massilia violacea* CAVIO and *Massilia agilis* J9, however MC02 still represents a distinct branch (Figure 9). The 16S rRNA sequences of *M. violacea* CAVIO and *M. agrilis* J9 only have a 96.79% and 96.72% identity to 16S rRNA of *M. arenosa,* respectively. Both are thereby lower than the typical 97% 16S rRNA similarity threshold that is typically applied to distinguish between different operational taxonomic units (OTU) or novel species (Stackebrandt & Goebel, 1994). Based on the phylogenetic tree and 16S rRNA identity percentages, *Massilia arenosa* MC02 is a novel species of the *Massilia* genus.

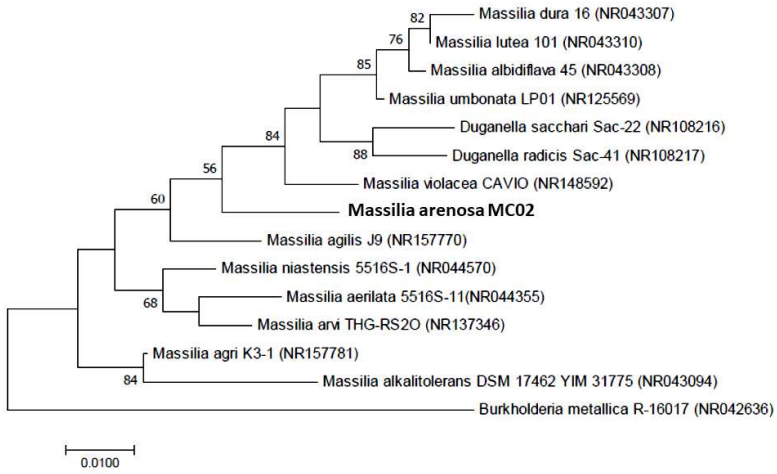

Figure 9. Maximum-likelihood phylogenetic tree created using MEGA7 based on the most similar 16S rRNA gene sequence analysis using MC02 as the query sequence. The numbers at the nodes indicate bootstrap values (based on 1000 replications). Bootstrap values above 50% were used.

Physiology and Chemotaxonomy

We used the Biolog Gen III MicroPlate test system (Biolog, Hayward, CA, USA) to characterize the metabolic capabilities of MC02. The 96-well plate has a positive and negative controls, 23 chemical sensitivity assays, and 71 carbon source utilization assays. The plates were inoculated with the appropriate inoculation fluid, and incubated for four days at 30°C. The plates were analyzed using the Biolog's Microbial Identification Systems software (e.g.OmniLog® Data Collection).

We compared the chemotaxonomic phenotype of *Massilia arenosa* MC02 with other members of the *Massilia* genus and other members of closely related genera of the *Oxalobacteraceae*. Using the 16S rRNA BLAST results, we selected fifteen bacterial isolates, based on similarity and availability in culture collections that represent a broad range of bacterial species from different genera within *Oxalobacteraceae*. Isolates were acquired from the American Type Culture Collection (ATCC, Manassas, USA), DSMZ (Braunscheig, Germany) and VTT Technical Research Centre of Finland (VTT, Finland). The bacterial species, and their accession information are available in Table 11 (Supplementary Material). All isolates were tested in accordance with the Biolog Gen III manufacturer manual (BIOLOG, 2013). To better visualize the results, a nonmetric multi-dimensional scaling (MDS) plot was created by converting the "+", "- ", "+/-" Biolog results (Supplementary Material, Table 12) into the numerical data 2, 0, 1 respectively. These results were then entered into R-studio to create nonmetric multidimensional (MDS) coordinates (Ross Ihaka & Robert Gentleman, 1996). According to this analysis, *M. arenosa* is not grouped with any of the bacterial strains and has also a distinctly different

chemotaxonomic phenotype than the other *Massilia* strains on the plot (Figure 10). Based on its metabolic and sensitivity testing, its chemotype is most similar to *Undibacterium terreum* C3 (DSMZ 102222) (Y. Q. Liu, Wang, Zhou, & Liu, 2013), *Oxalicibacterium faecigallinarum* YOx (DSMZ 21641) (Sahin, Portillo, Kato, & Schumann, 2009), *Herminiimonas contaminans* CCUG 53591 (DSMZ 28178) (Kampfer, Glaeser, Lodders, Busse, & Falsen, 2013), and other *Massilia* species.

The main differences between *M. arenosa* and the four other *Massilia* species; *M. albidiflava* 45 (DSMZ 17472), *M. aerilata* 5516S-11 (DSMZ 19289)*, M. umbonata* LP01 (DSMZ 26121), and *M. dura* 16 (DSMZ 17513) are listed in Table 8. In contrast to some of the other *Massilia* species, *M. arenosa* can utilize D-turanose, inosine, L-glutamic acid and L-serine. Consistent with the utilization of inosine, we identified in the genome of MC02, the gene for a putative inosine-5'-monophosphate dehydrogenase, which catalyzes the first rate-limiting step in the synthesis of guanin nucleotides from inosine-5'-monophosphate (Pankiewicz & Goldstein, 2003). We also found in the MC02 genome, genes encoding a putative dihydrofolate synthase/folylpolyglutamate synthase that is involved in L-glutamic acid utilization (Banerjee, Shane, McGuire, & Coward, 1988). In contrast to other *Massilia* strains, *M. arenosa* MC02 cannot utilize substrates, such as sucrose, D-melibiose, D-galactose, gelatin, and L-aspartic acid. To break down sucrose into glucose and fructose, invertase or β-D-fructofuranoside fructohydrolase activities are required (Lincoln & More, 2017). Consistently, the genome of MC02 lacks the genes for these enzymes. Similarly, MC02 also does not possess an α-galactosidase gene to hydrolyze D-melibiose (R. L. Zhao et al., 2018). In contrast to several of the other Massilia

strains, MC02 showed resistance against the two antibiotics vancomycin and rifamycin SV, that inhibit the cell wall biosynthesis and the RNA polymerase binding site, respectively (Molodtsov, Scharf, Stefan, Garcia, & Murakami, 2017; Sarkar, Yarlagadda, Ghosh, & Haldar, 2017).

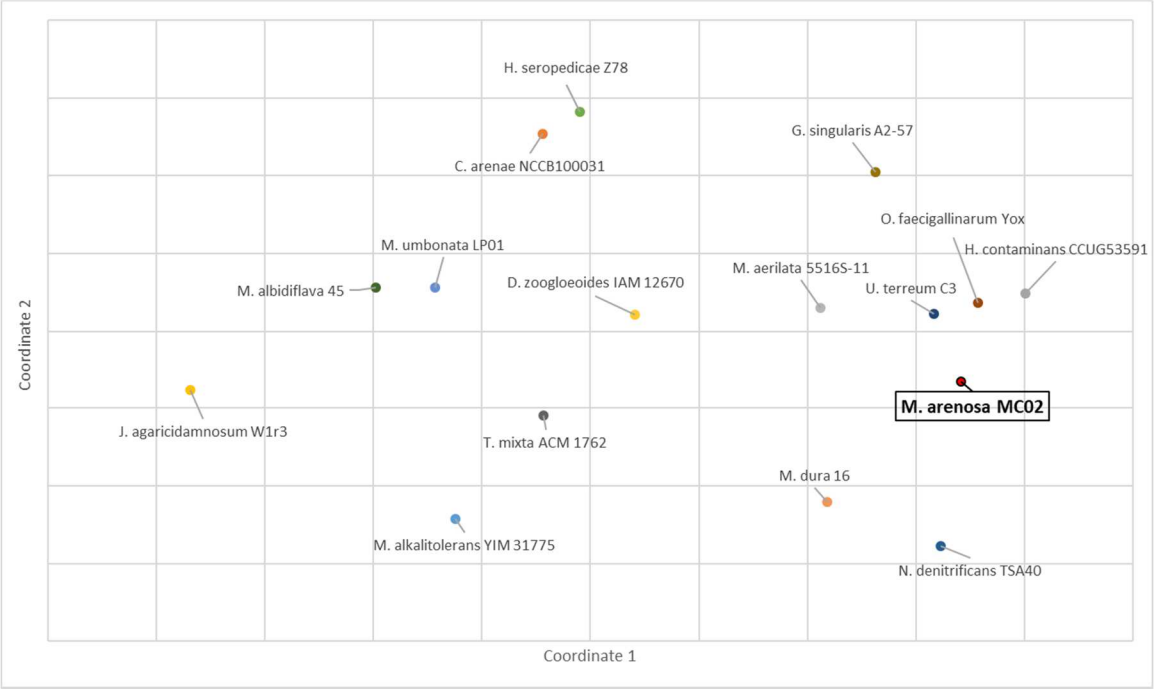

Figure 10. Non-metric multidimensional scaling plot based on the Biolog results of MC02 and similar genera from a 16S rRNA BLAST and availability in culture collections. Isolate information in supplemental Table 11.

Table 8. Differences in the utilization of different substrates and in the resistance against different antibiotics between *M. arenosa* MC02, and four *Massilia* strains: *M. albidiflava* 45, *M. aerilata* 5516S-11, *M. umbonata* LP01, and *M. dura* 16 based on Biolog assays. The reference strains and their numbers in culture collections are provided in Supplemental Table 11. All plates were incubated for four days, and the appropriate growth medium and inoculation fluid is indicated in the Supplemental Table 12.

Biochemical tests	*Massilia arenosa*	*Massilia albidiflava*	*Massilia aerilata*	*Massilia umbonata*	*Massilia dura*
Description	MC02	DSMZ 17472	DSMZ 19289	DSMZ 26121	DSMZ 17513
Inoculation-fluid	B	A	A	A	B
Growth Medium	R2A	R2A	R2A	R2A	TSA
Negative Control	-	-	-	-	-
Positive Control	+	+	+	+	+
Utilization of:					
Sucrose	-	+	-	+	+
D-Turanose	+	-	±	-	+
D-Melibiose	-	+	-	+	-
D-Galactose	-	+	+	+	+
Inosine	+	-	-	-	+
Gelatin	-	+	+	+	-
L-Aspartic Acid	-	+	+	+	-
L-Glutamic Acid	+	+	+	+	-
L-Serine	+	+	-	-	-
Resistance to:					
Vancomycin	+	-	-	-	±
Rifamycin SV	+	-	+	+	+

MALDI-TOF

Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) was used to further confirm that MC02 is a novel species (Singhal et al., 2015). The MALDI-TOF spectra were obtained using the Bruker microflex instrument (Bruker Daltonics Inc., Billerica, MA), the direct transfer protocol supplied by the manufacturer, and the FlexAnalysis v3.4 software. MC02 was grown overnight and a single colony was used for evaluation. MC02 cells, as well as a bacterial test standard, were spread on a polished steel target plate and treated with formic acid. The cells were then applied to the matrix, α-Cyano-4-hydroxycinnaminc acid (HCCA). Once the plate

dried, the spectra were collected over a 2 to 20 kiloDalton range. Two replicate measurements were conducted.

Once the spectra were obtained, we compared the MC02 spectra against the database of reference spectra. The flexAnalysis software allowed to calculate a spectra similarity score, and the log of this score was used to determine a strain similarity estimate. A score greater than 2.0 is generally considered to be a match at the species level, a score of 1.7 to 2.0 represents a match at the genus level, and a score below 1.7 suggests a novel species (Croxatto, Prod'hom, & Greub, 2012). When *M. arenosa* MC02 was compared against itself as a control and compared against other bacterial species in the database, the low similarity score of 1.23 with the most closely related species *Massilia aerilata* DSM19289 in the database, also confirms that MC02 is a novel species.

Genome Features

Genomic DNA was sequenced as previously described using Illumina NextSeq. The Genome was assembled using SPAdes 3.11.0 (Bankevich et al., 2012) and the assembly quality was analyzed using BUSCO (Simao et al., 2015) which revealed a measured completeness of 95%. We obtained 1,566,408 total reads with a total read length of 231,096,882 bp and an average read length of 148 bp. Genome assembly and annotation were carried out by PATRIC 3.5.27 (Wattam et al., 2017) which resulted in 275 contigs, and confirmed by the RAST platform (Overbeek et al., 2005). According to this pipeline, the genome length of *Massilia arenosa* MC02 was 5,023,356 bp. We identified a total of 4,790 protein coding sequences consisting of 1,762 hypothetical proteins and 3,028

proteins with functional assignments. There was a total of 47 tRNA genes and 2 rRNA operons and a N50 value of 28,267 (range: 1,074 - 119,695 bp) and L50 value of 49. The percent GC content was 66.2%.

Several genes with putative plant-growth promoting capabilities were identified in the MC02 genome by annotation with Galaxy(Afgan et al., 2018). The MC02 genome contains genes for nitrate reductase (*napA*), several phosphatase genes (*ppk, phoA, phoB, phoD, phoR*), and biotin biosynthesis genes (b*ioA, bioB, bioD, bioF*). Using RAST 2.0 (Overbeek et al., 2005) we also identified 48 putative virulence genes, including 34 genes indicating a resistance to antibiotics and toxic compounds. MC02 also has putative endophyte genes comprising of 14 genes involved in invasion and intracellular resistance, and 26 genes responsible for flagellar motility (Czaban, Gajda, & Wroblewska, 2007).

The Microbial Genomes Atlas (MiGA) was used to identify the closest related genomes from other species and to determine if MC02 represents a novel species (M. et al., 2018). Using the prokaryotic pipeline of MiGA, the closest related strain to MC02 from the NCBI database is *Massilia armeniaca* NZ CP028324 with an average amino acid identity (AAI) of 67.37%. This low AAI is just above 65%, the threshold that MiGA applies to predict a new genus. P-values are used to reflect the confidence for each taxonomic rank assignment, and MC02 has a p-value of 0.215 for the genus and 0.488 for the species level, respectively. P-values are also used to determine the taxonomic novelty of the isolate and to show the probability of any two genomes in the NCBI RefSeq database of having an AAI less than or equal to the query AAI value. MiGA shows a novelty p-value of 0.48 at the genus level, and 0.00847 at the species level. In addition to the low AAI value

with *M. armeniaca* NZ CP028324, the high taxonomic p-value indicates that MC02 does not belong to the same species as the closest related genome, and the low novelty p-value confirm also on the genome level that *M. arenosa* MC02 is a novel species within the *Massilia* genus.

We also utilized EZBioCloud (Yoon, Ha, Kwon, et al., 2017) to calculate Average Nucleotide Identity by Orthology (OrthoANI) (Yoon, Ha, Lim, et al., 2017) values and to confirm that MC02 represents a new species. OrthoANI identifies the reciprocal best hits (orthologous relationship) of a pair of fragments, by running reciprocal BLASTn searches with each fragment (Lee, Kim, Park, & Chun, 2016). The OrthoANI values to the closest full reference genomes are between 76.4 and 77.1% (Table 9), which and much lower than the 95-96% new species cutoff boundary for bacteria (Goris et al., 2007).

Table 9. OrthoANI values from EzBioCloud based on *Massilia arenosa* MC02 as the query genome and the closest full reference genomes.

Isolate	OrthoANI value (%)
Massilia sp. WG5	77.14
Massilia sp. NR 4-1	76.5
Massilia armeniaca strainZMN 3	76.38
Massilia sp. YMA	76.35

Genome-to-genome-distance-calculations (GGDC) measure intergenomic distance and use three different formulas to predict the DDH probability (Meier-Kolthoff et al., 2013). When MC02 is compared to the four most similar full genomes, based on BLAST results from the largest contig, the DDH probability for all three formulas and all four genomes is 0%, and lower than the ≥70% threshold to identify a novel species

(Table 10) (A. F. Auch, Klenk, & Goker, 2010). This is also enforced by the model confidence intervals.

Table 10. GGDC values based on MC02 as the query, compared to the four most genetically similar strains.

Formula 1 (High-scoring segment pairs Length / Total Length)					
Query genome	Reference genome	DDH	Model C.I.	Distance	Prob. DDH >= 70%
Massilia arenosa MC02	Massilia armeniaca strainZMN 3	19.1	[15.9 - 22.6%]	0.7381	0
Massilia arenosa MC02	Massilia sp. NR 4-1	18.5	[15.4 - 22.1%]	0.7539	0
Massilia arenosa MC02	Massilia sp. WG5	21.3	[18.1 - 25%]	0.6773	0
Massilia arenosa MC02	Massilia sp. YMA	19.3	[16.1 - 22.9%]	0.7312	0
Formula 2 (Identities / High-scoring segment pairs Length)					
Query genome	Reference genome	DDH	Model C.I.	Distance	Prob. DDH >= 70%
Massilia arenosa MC02	Massilia armeniaca strainZMN 3	21	[18.7 - 23.4%]	0.2096	0
Massilia arenosa MC02	Massilia sp. NR 4-1	20.8	[18.6 - 23.2%]	0.2113	0
Massilia arenosa MC02	Massilia sp. WG5	21	[18.8 - 23.4%]	0.2092	0
Massilia arenosa MC02	Massilia sp. YMA	21	[18.8 - 23.4%]	0.209	0
Formula 3 (Identities / Total Length)					
Query genome	Reference genome	DDH	Model C.I.	Distance	Prob. DDH >= 70%
Massilia arenosa MC02	Massilia armeniaca strainZMN 3	18.5	[15.9 - 21.6%]	0.793	0
Massilia arenosa MC02	Massilia sp. NR 4-1	18.1	[15.4 - 21.1%]	0.8059	0
Massilia arenosa MC02	Massilia sp. WG5	20.4	[17.6 - 23.4%]	0.7448	0
Massilia arenosa MC02	Massilia sp. YMA	18.7	[16.1 - 21.8%]	0.7874	0

Proposal of Massilia arenosa sp. nov. MC02

Based on the morphological, biochemical, phylogenetic and genomic analysis, it can be concluded that *Massilia arenosa* MC02 is a novel species of the *Massilia* genus. Compared to the reference strains *M. dura* 16 and *M. aerilata 5516S-11* that form relatively fast-growing yellow colonies, *M. arenosa* grows slower and forms cream colored colonies (Figure 7). The chemotaxonomic phenotype of MC02 differs in its ability to utilize different carbon and nitrogen substrates, and its resistance against the two antibiotics Vancomycin and Rifamycein SV from reference strains of the *Massilia* genus (Table 8). The MALDI-TOF results indicated that *M. arenosa* is most similar to M. *aerilata* DSM19289, however with a score of 1.23 which is below the new isolate

threshold of 1.7, indicating that MC02 is most likely a new species. The analysis of AAI, OrthoANI, and GGDC to the most similar reference strains supports the conclusion that *Massilia arenosa* MC02 is a novel species.

Description of M. arenosa sp. nov.

Massilia arenosa sp. nov. **(**arēnōsa. L. part. adj. sandy, as it was isolated from a sandy loam soil). *Massilia arenosa* sp. nov. was isolated from a soil sample from a maize field in California with the geographical coordinates 37.6058, -120.7478. The field was classified as Hanford sandy loam, and earthy peat soil. It grows on R2A media, with a temperature range of 21-30°C. Its colony morphology on R2A is circular, flat, with entire margins, pinpoint to medium in size, and cream colored. Cells are gram negative rods in singles or doubles. Based on the Biolog results, it can grow at a pH of 6 but not at pH 5. A salt concentration of 1% and higher inhibits the growth. *M. arenosa* can metabolize sugars, such as dextrin, D-maltose, D-trehalose, D-cellobiose, gentiobiose, D-turanose, a-D-glucose, D-mannose, and inosine, and utilize various amino acids, such as L-glutamic acid, L-serine, and b-hydroxy-D, L-butyric acid. *M. arenosa* MC02 is resistant to antibiotics, such as rifamycin SV, vancomycin, and aztreonam. MC02 contains the following benificial genes; nitrate reductase (napA), phosphatase genes (ppk, phoA, phoB, phoD, phoR), and biotin biosynthesis genes (b*ioA, bioB, bioD, bioF*). As well as, 48 putative virulence genes, 34 antibiotic resistance genes, 14 invasion and intracellular resistance genes, and 26 flagella motility genes. The genome is 5.02 Mbp and the GC content is 66.2%.

Table 11. Accession number and culture collection information of the used reference strains to MC02.

Isolate	Accession number	Culture Collection
Collimonas arenae strain NCCB 100031	NR_042824.1	DSMZ 21398
Duganella zoogloeoides strain IAM 12670	NR_025833.1	ATCC 25935
Glaciimonas singularis strain A2-57	NR_109670.1	DSMZ 100199
Herbaspirillum seropedicae Z 78	AJ238361	DSMZ 6446
Herminiimonas contaminans strain CCUG 53591	NR_108871.1	DSMZ 28178
Janthinobacterium agaricidamnosum strain W1r3	NR_026364.1	DSMZ 9628
Massilia aerilata strain 5516S-11	NR_044355.1	DSMZ 19289
Massilia albidiflava 45	AY965999	DSMZ 17472
Massilia alkalitolerans YIM 31775	NR_0430941	VTT 032361
Massilia dura strain 16	NR_043307.1	DSMZ 17513
Massilia umbonata strain LP01	NR_125569.1	DSMZ 26121
Noviherbaspirillum denitrificans TSA40	NR_157007.1	ATCC TSD-69
Oxalicibacterium faecigallinarum strain YOx	NR_112834.1	DSMZ 21641
Telluria mixta strain ACM 1762	NR_044833.1	ATCC 49107
Undibacterium terreum strain C3	NR_109599.1	DSMZ 102222

Table 12. Biolog results for *Massila arenoa* MC02 and all comparable species from supplemental Table 11. Plates were incubated for four days and the growth medium and inoculation fluid is identified on the table.

Biochemical Test	*Massilia arenose*	*Massilia albidiflava*	*Collimonas arenae*	*Herminiimonas contaminans*	*Jeotkiaoubacterium agaricidammarum*	*Naxibacter (Massilia) alkalitolerans*	*Herbaspirillum serapedicae*	*Duganella zoogloeoides*	*Undibacterium terr cum*	*Oxalicibacterium f aecigallinarum*	*Telluria mixta*	*Glaciimonas singularis*	*Massilia aerilata*	*Massilia amboinia*	*Naxibarbaspirillum denitrificans*	*Massilia dura*
Reactions	014VDT	DSMZ 17472	DSMZ 21398	DSMZ 28178	DSMZ 9628	VTT 032361	DSMZ 6446	ATCC 25935	DSMZ 102222	DSMZ 21641	ATCC 49107	DSMZ 100199	DSMZ 19289	DSMZ 26121	ATCC TSD-69	DSMZ 17513
Inoculation Fluid	B	A	A	A	A	A	B	A	B	A	B	A	A	A	B	B
Media	R2A	R2A	Nutrient	TSA	Nutrient	TSB	Nutrient	TSA	R2A	Nutrient	Nutrient	R2A	R2A	R2A	R2A	TSA
Negative Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dextrin	+	+	-	-	+	±	±	+	-	-	+	-	+	+	+	+
D-Maltose	+	+	-	-	+	±	-	+	-	-	+	-	+	+	+	+
D-Trehalose	+	+	-	+	+	+	-	+	-	-	+	-	-	+	+	+
D-Cellobiose	+	+	-	-	+	+	-	+	-	-	±	-	+	+	-	+
Gentiobiose	+	+	-	+	+	+	-	+	-	+	-	-	+	+	-	+
Sucrose	-	+	-	-	+	+	-	-	-	-	-	-	-	+	+	+
D-Turanose	+	-	-	-	+	±	-	-	-	-	-	-	±	-	+	+
Stachyose	-	-	-	-	+	+	-	-	-	-	-	-	-	+	+	-
Positive Control	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH 6	+	+	+	-	+	+	+	+	-	±	+	+	+	+	+	+
pH 5	-	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-
D-raffinose	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-	-
a-D-Lactose	-	+	-	±	+	-	-	-	-	-	-	-	-	±	+	-
D-Melibiose	-	+	-	-	+	±	-	-	-	-	-	-	-	+	+	-
B-Methyl-D-Glucoside	-	+	-	-	+	+	-	-	-	-	+	-	-	-	-	-
D-Salicin	-	+	-	-	+	+	-	+	-	-	+	-	-	±	-	-
N-Acetyl-D-Glucosamine	-	+	+	-	+	+	±	-	-	-	+	+	-	-	+	-
N-Acetyl-B-D-Manosamine	-	-	-	-	+	-	-	-	-	-	±	-	-	-	+	-
N-Acetyl-D- Galactosamine	-	+	-	-	+	-	-	-	-	-	±	-	-	+	-	-
N-Acetyl-D- Galactosamine	-	-	-	-	+	-	-	-	-	-	±	-	-	-	-	-
1% NaCl	-	-	-	-	+	+	+	+	-	+	+	-	-	-	+	+
4% NaCl	-	-	-	-	+	+	±	-	-	-	+	-	-	-	-	-
8% NaCl	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
a-D-glucose	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+
D-Mannose	+	+	+	-	+	+	±	+	-	-	-	-	+	+	+	+
D- Fructose	-	+	+	-	+	+	±	+	-	-	+	±	-	+	+	+
D-Galactose	-	+	+	-	+	+	+	+	+	-	±	-	+	+	+	+
3-Methyl Glucose	-	+	±	-	+	±	-	-	-	-	±	-	-	+	-	-
D-Fucose	-	+	+	-	+	-	±	-	-	-	±	-	-	±	-	-
L-Fucose	-	+	+	-	+	-	+	±	-	-	±	-	-	+	-	-
L-Rhamnose	-	+	-	-	+	+	+	+	-	-	±	-	±	+	-	+
Inosine	+	-	+	-	+	+	-	+	-	-	+	-	-	-	-	+
1% Sodium Lactate	-	-	+	+	+	+	+	+	-	+	+	±	-	+	+	-
Fusidic Acid	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
D-Serine	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	±
D-Sorbitol	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	+
D-Mannitol	-	-	+	-	+	+	+	-	-	-	±	-	-	+	+	+
D-Arabitol	-	±	+	-	+	±	+	-	-	-	±	-	-	±	-	-
Myo-Inositol	-	+	+	-	+	+	+	-	-	-	+	+	-	±	-	-
Glycerol	-	-	+	-	+	+	+	+	-	-	+	+	-	+	+	+
D-Glucose-6-PO4	-	+	+	-	+	-	±	±	+	-	+	-	+	+	+	-
D-Fructose-6-PO4	-	+	+	-	+	±	±	±	-	-	+	-	±	+	+	-
D-Aspartic Acid	-	-	+	-	+	+	+	-	-	-	-	±	-	-	-	-
D-Serine	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-
Troleandomycin	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-
Rifamycin SV	+	-	+	+	+	+	+	+	-	+	-	+	+	+	-	+
Minocycline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin	-	+	±	-	+	+	-	+	-	-	+	-	+	+	-	-
Glycyl-L-Proline	-	+	+	-	+	+	+	+	+	+	±	-	-	+	-	-
L-Alanine	-	+	+	-	+	+	±	+	-	-	-	+	-	+	-	-
L-Arginine	-	+	±	-	+	+	-	+	-	-	±	+	-	±	-	-
L-Aspartic Acid	-	+	+	-	+	+	+	+	-	-	+	+	+	+	-	-
L-Glutamic Acid	+	+	+	-	+	+	+	+	+	±	+	+	+	+	-	-
L-Histidine	-	+	+	-	+	+	-	+	-	-	±	+	-	±	-	-
L-Pyroglutamic Acid	-	+	+	-	+	±	+	-	-	-	±	-	-	-	-	-
L-Serine	+	+	+	-	+	+	-	±	-	-	+	+	-	-	-	-
Linocomycin	+	+	+	-	-	+	+	+	+	+	-	+	+	+	-	+
Guanidine HCl	-	-	-	-	+	+	+	-	-	-	+	-	-	-	-	+
Niaproof 4	-	-	-	-	-	-	±	-	-	-	-	-	-	-	-	-
Pectin	-	+	-	-	+	+	-	+	-	-	-	-	-	+	+	+
D-Galacturonic Acid	-	+	-	-	+	-	+	+	-	-	-	-	+	+	-	-
L-Galactonic Acid Lactone	-	+	±	-	+	-	±	-	-	-	-	-	+	+	-	-
D-Gluconic Acid	-	+	+	-	+	+	+	+	+	-	+	-	-	+	-	+
D-Glucuronic Acid	-	+	+	-	+	+	+	+	-	-	-	-	-	+	-	-
Glucuronamide	-	+	+	+	+	-	±	±	-	±	±	-	-	+	-	-
Muric Acid	-	+	+	-	+	±	+	+	-	-	±	-	+	-	-	-
Quinic Acid	-	-	+	-	+	+	+	-	-	-	±	+	-	+	-	-
D-Saccharic Acid	-	+	+	-	+	+	+	+	-	-	±	-	+	+	-	-
Vancomycin	+	-	+	+	-	+	+	-	-	+	-	+	-	-	-	±
Tetrazolium Violet	-	-	+	-	-	-	+	-	-	-	-	-	-	+	-	±
Tetrazolium Blue	-	+	+	-	+	-	+	±	-	+	-	+	-	+	-	-
p-Hydroxy-Phenylacetic Acid	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-
Methyl Pyruvate	+	+	+	+	±	±	+	+	-	+	+	+	+	±	-	+
D-Lactic Acid Methyl Ester	-	±	-	-	+	-	-	-	-	-	±	-	-	-	-	-
L-Lactic Acid	-	+	+	+	+	±	+	-	+	+	+	+	-	+	-	+
Citric Acid	-	-	+	-	+	+	+	+	+	-	-	-	+	+	-	-
a-Keto-Glutaric Acid	-	+	+	-	+	-	+	±	+	+	+	-	+	+	-	+
D-Malic Acid	-	+	-	-	+	-	+	-	-	-	±	-	+	+	-	-
L-Malic Acid	-	±	+	+	+	+	+	+	+	+	+	-	+	+	-	-
Bromo-Succinic Acid	-	+	±	±	+	+	+	+	-	-	+	-	-	+	-	-
Nalidixic Acid	-	-	+	±	-	+	+	-	-	-	-	-	+	-	-	+
Lithium Chloride	-	-	-	-	+	+	-	-	-	-	+	-	-	-	+	+
Potassium Tellurite	-	-	±	-	+	+	+	-	-	-	+	+	-	-	+	+
Tween 40	+	+	+	-	+	±	-	±	±	-	+	-	+	+	-	-
y-Amino-Butyric Acid	-	-	+	-	+	+	+	-	-	-	-	+	-	-	-	-
a-Hydroxy-Butyric Acid	-	-	±	-	±	-	±	-	-	-	-	±	-	±	-	+
b-Hydroxy-D, L-Butyric Acid	+	+	+	+	±	-	+	+	-	-	±	+	+	+	-	-
a-Keto-Butyric Acid	-	+	+	-	±	-	+	+	-	-	±	±	-	+	-	+
Acetoacetic Acid	-	-	-	±	+	+	±	±	±	-	+	+	±	+	-	+
Propionic Acid	-	-	+	+	+	-	±	+	-	-	±	+	±	+	-	-
Acetic Acid	-	-	+	+	+	-	+	+	+	+	+	+	+	+	-	-
Formic Acid	-	-	+	+	±	-	+	-	±	-	+	-	-	-	-	-
Aztreonam	+	+	-	-	+	+	+	+	+	+	+	-	+	+	-	+
Sodium Butyrate	-	-	-	-	+	+	±	±	-	±	+	-	-	-	+	+
Sodium Bromate	-	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-

Author Statments

Author statement

All authors approved the submission of this manuscript to the International Journal of Systematic and Evolutionary Microbiology. The authors contributed as follows: RR and VP conducted the experiments, RR, VP and HB conceptualized the project, analyzed the experiments, and validated the results, and wrote, reviewed and edited the manuscript,

HB supervised the experiments, and was responsible for the acquisition of the financial support.

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Ethical statement

No experimental work that included human or animal subjects was conducted.

Conflicts of interest

The authors declare that there are no conflicts of interest.

CHAPTER 4

Draft Genome of *Massilia* sp. nov., a Novel Bacterial Species of the Oxalobacteraceae family

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Running title: Massilia hortus
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Abstract

We isolated from garden soil and sequenced a new member of the Family *Oxalobacteraceae* Genus *Massilia*: *Massilla* sp. ONC3. Sequence analysis showed an assembled genome size of 5,622,601 bp with a predicted total of 5,104 protein coding sequences, 3,194 functionally assigned genes, 2 *rRNAs*, and 56 *tRNAs*.

Announcement

Members of the genus *Massilia* have been isolated from soil, air and water samples as well as from the plant rhizosphere and endosphere, and are typically Gram negative, rod shaped aerobes (Maya et al., 2012; Vikram, Govender, Kabwe, Bezuidt, & Makhalanyane, 2017). Certain species of *Massilia* can promote plant growth through their ability to solubilize recalcitrant phosphate sources in soils (B. X. Zheng, Bi, Hao, Zhou, & Yang, 2017). *Massilia* species can also have a positive impact on the colonization of plants by beneficial root symbionts, such as arbuscular mycorrhizal fungi. *Massilia* sp. RK4, for example, increases root colonization by arbuscular mycorrhizal fungi and their nutritional benefits for maize plants under salt stress (Krishnamoorthy et al., 2016). Other *Massilia*

species are highly resistant to heavy metals and have been isolated from mines (Feng, Yang, Li, & Zhu, 2016).

We isolated a putative new species of *Massilia* from unplanted garden soil with a pH of 5.2 near Maxton, North Carolina, US (34.6494, -79.4327). The soil samples were dried for 3 days prior to bacterial isolation, then mixed with 1X PBS solution, bacterial colonies were streaked out several times before isolating single colonies on R2A media at 30°C. Cultures were grown in R2A broth at 30°C for 2 days and genomic DNA was extracted using the AllPrep Bacterial DNA/RNA/Protein kit (QIAGEN Inc., Germantown, MD) and the protocol for bacterial gDNA extraction. The genomic library was prepared using the Illumina Nextera platform (San Diego, CA), size selected to an average fragment length of 475 bp and sequenced using the Illumina NextSeq paired-end v2 chemistry on v2.5 flowcells at 150 bp per read. Target coverage for reads was 20x. Genome assembly using SPAdes 3.13.0 (Bankevich et al., 2012) resulted in 155 contigs, with an N_{50} value of 56,531 bp (range: 942 to 207,795 bp) and a total assembled size of 5,622,601 bp with a GC content of 63.82%. Assembly quality through BUSCO (Simao et al., 2015) revealed a measured completeness (40 single copy BUSCO's) of 100%. The assembly was annotated with PATRIC (Wattam et al., 2017) annotation pipeline. Galaxy platform, PATRIC 3.5.28 and RAST 2.0 (Aziz et al., 2008). which identified of 5,104 protein coding sequences, 3,194 proteins with functional assignments, 2 rRNA operons, and 56 tRNA genes.

Based on a NCBI BLAST (Altschul S.F., 1990) searches of the 16S rRNA, *Massilla sp. ONC3* has the highest similarity (98% sequence identity) with *Massilia solisilavae* J18 and *Massilia terrae* J11. The genome to genome distance calculator (GGDC) (Meier-Kolthoff

et al., 2013) was compared to the 5 most related genomes (*Massilia albidiflava* strain DSM 17472, *Massilia armeniaca* strain ZMN-3, *Massilia putida* strain 6NM-7T, *Massilia sp*. NR 4-1, and *Massilia sp*. WG5). The GGDC, which calculates intergenomic distance using three formulas was also used. To generate percentage matches, the sum of all identities found in aligned high-scoring segment pairs was then divided by the total genome length. The results for the ONC3 genome relative to the other selected genomes showed a 0% match (or less than 70% of the scientific community threshold) (Tindall et al., 2010; Wayne et al., 1987). This suggests that *Massilla* sp. strain ONC3 is a novel species within the genus *Massilia*.

Data availability

The genome has been deposited at DDBJ/EMBL/GenBank under the BioProject number: PRJNA529408 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA529408), BioSample number: SAMN11265771, and accession number: SPUM00000000.

Acknowledgements

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Massilia hortus sp. nov, strain ONC3 a novel species of *Massilia* isolated from garden soil

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Abbreviations: ML, maximum-likelihood, AAI, average amino acid index, DSMZ, German Collection of Microorganisms and Cell Cultures GmbH

The GenBank/EMBL/DDBJ accession numbers for this genome of Massilia hortus sp. nov strain ONC3 are listed under the BioProject number: PRJNA529408 (BioSample number: SAMN11265771 and Accession number: SPUM00000000).

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Abstract

A novel member of the *Massilia* genus was isolated from garden soil in North Carolina, was designated strain ONC3. Using complete 16S *rRNA* sequences a phylogenetic analysis and full genome analysis was performed. The results supported ONC3 being a novel species of the genus *Massilia.* Close family members when comparing 16S *rRNA* are: *Massilia solisilvae* strain J18, *Massilia terrae* strain J11 and *Massilia agilis* strain J9 (97.80, 97.72 and 97.25 percent identity, respectively). Chemotaxonomic testing using the Biolog Gen III microplates, showed the ONC3 did not cluster with any close type strains. Average amino acid index (AAI) genome comparison revealed that *Massilia putida* NZ CP019038 had an AAI of 73.06%. The Genome-Genome distance calculator (GGDC) showed a 0% DNA-DNA hybridization greater than or equal to 70. The results from chemotaxonomic, phylogenetic, genome and physiological analysis, show that ONC3 should be considered

a novel species of *Massilia* and the name of *Massilia hortus sp. nov*. strain ONC3 is proposed.

Introduction

The genus of *Massilia* belongs to the family *Oxalobacteraceae* and the class of Betaproteobacteria, and consists of 26 species of bacteria (José Ivo Baldani et al., 2014). The members of this genus are Gram negative, aerobic rods that are motile (Feng et al., 2016). The motility originates from the presence of a polar flagellum, but species with flagella on the lateral sides of the cell have also been described (Y. Q. Zhang et al., 2006) . The first member of the genus, *Massilia timonae was* originally isolated from human blood samples (B. La Scola, Birtles, Mallet, & Raoult, 1998), but members of the *Massilia* genus have also been isolated from various environmental sources such as air, soils, aerosols, freshwater, the rhizosphere and plant roots plants (Ofek et al., 2012). In the rhizosphere of soybean plants, *Massilia* species can account for 6% of the bacterial community (Sugiyama, Ueda, Zushi, Takase, & Yazaki, 2014). Some *Massilia* species have plant growth promoting capabilities for example solubilize phosphate in actively farmed and fertilized soils (B. X. Zheng, Bi, et al., 2017). Massilia have also been isolated from zinc and lead contaminated soils and produces indole-3-acetic acid and could be potentially used for reclamation or planting in contaminated soils (Kuffner et al., 2010).

This investigation was performed to access the novelty of a bacterial strain that had been isolated from garden soil near Maxton, North Carolina. This strain is associated the genus *Massilia*, however, differs from known and characterized species. 16S *rRNA* analysis provided a definite genus taxonomic identification to *Massilia*, but

the species designation could not be ascertained by this and thus additional phenotypic and genotypic analysis was performed. From these results we have determined that the isolate ONC3 is of the *Massilia* genera but is of a novel specie. We propose to name this strain, *Massilia hortus sp. nov*. strain ONC3.

Isolation and ecology

ONC3 was isolated from an unplanted garden soil with a pH of 5.2 near the town of Maxton (North Carolina, USA; 34.6494, -79.4327). The environment of the sampling area has a mean annual precipitation of 38-55 inches and has a mean annual air temperature of 59-70°F. The soil type is predominately a loamy sand soil [8, 9]. The soil was first suspended in a phosphate buffered saline solution, and after plating an aliquot of the mixture on soil extract medium, ONC3 was streaked for isolation of single cell colonies and grown at 30°C (Subba Rao, 1977). After colony isolation, ONC3 was cultured and maintained on R2A agar at 30°C (Reasoner & Geldreich, 1985). Single cell isolation was repeated to confirm purity of the isolate which was then transferred to a 20% glycerol solution and stored at -80°C for downstream processing.

Phenotypic Characterization

Cells of *M. hortus* strain ONC3 are Gram negative rods and show a chain like cellular arrangement. When cultured on R2A medium, ONC3 forms light brown, circular, flat colonies. We performed a chemotaxonomic characterization by comparing ONC3 with 15 reference strains from the *Massilia* genus (Table 11). The reference strains were selected based on a 16S *rRNA* BLAST search and were obtained from the American Type

Culture Collection (ATCC, Manassas, VA, USA) or the Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures (DMSZ, Braunscheig, Germany). The strains were then cultured on the respective culture media recommended by the culture collection (R2A or Tryptic Soy Agar, see also Table 13) and were then chemotaxonomically characterized using the Gen III Microlog System (Biolog, Hayward, CA ,USA) which utilizes a 96-well plate that contains 94 phenotypic tests; 71 substrate utilization tests and 23 chemical sensitivity tests (Bochner, 1989). For the Microlog analysis, the strains were briefly grown on the required media for 2 days at 30°C, and then transferred into an inoculation fluid that was supplied with the Biolog plates and then standardized to an optical density of 98%. This solution was then deposited in 100 µL increments into the wells of the plate which was then incubated for 2 days. The plates were then read, and the results were entered into the Microlog system as follows: + for positive, - for a negative, and +/- for a borderline result, and then converted into the number values 2, 0, or 1, respectively.

The number results were processed in R-studio (R. Ihaka & R. Gentleman, 1996) to generate a non-metric multidimensional scaling plot and to observe any clustering of the bacterial strains (Fig. 1). Based on similar chemotaxonomic profiles, ONC3 was positioned closest, yet was not clustered with *Undibacterium terreum* strain LP01, Biolog results also showed that ONC3 could utilize histidine as a nitrogen source whereas, only *Massilia albidiflava* strain 45 showed histidine utilization. ONC3 also has histidine regulating genes, such as the *hutH* and *hutU* which are a part of the *HutP* operon that regulate histidine utilization (Bender, 2012). However, *M. albidiflava's* Biolog profile did

not match that of ONC3 which also provides support that these strains are different from each other. Literature suggests that only certain species of *Massilia* can utilize histidine, and even fewer species use it to produce Violacein, a antibacterial and cancer agent (Agematu, Suzuki, & Tsuya, 2011b; Cho et al., 2017; Seong Yeol Choi, Yoon, Lee, & Mitchell, 2015). However, ONC3 does not show the phenotypic or genetic qualities of being able to produce violacein. The data shows that this isolate's chemotaxonomic and Biolog profile is different than those of related species under the *Massilia* genus.

Table 13. Bacterial isolates purchased from culture collection banks and their corresponding catalog codes and culturing media.

Bacterial Isolate	**Culture Collection Catalog Code**	**Culture Collection**	**Culturing Media (DSMZ Recipe)**
Massilia aerilata	19289	DSMZ	R2A
Massilia albidiflava	17472	DSMZ	R2A
Massilia alkalitolerans	VTT:CAS29	VTT	R2A/ Tryptic Soy Agar
Massilia dura	17513	DSMZ	R2A
Massilia namucuonensis	2159	DSMZ	R2A
Massilia umbonata	26121	DSMZ	R2A

Figure 11. Non-metric multidimensional plot of ONC3 and similar *Massilia* genera members based on the Biolog results.

MADLI-TOF MS

Matrix-assisted laser desorption/ionization time of flight mass spectroscopy (MALDI-TOF MS) was performed by Novozymes A/S. MALDI-TOF can rapidly characterize microorganisms by utilizing a target plate was prepared by mixing bacterial cell samples and matrix solution and dried to trap the samples in place. The sample matrix is then ionized with a laser beam which accelerates the ions at fixed speed or flight. For microbial analysis, time of flight is used to measure the mass-to-charge ratio (m/z) of ions by determining the length of time an ion takes to go through the flight tube of the mass spectrometer. A spectrum is made that displays peptide mass fingerprint of the

bacterial isolate (Holland et al., 1996; Singhal et al., 2015). MALDI-TOF was carried out on a Bruker microflex instrument, running the flexControl v3.4 software (Bruker Daltonics Inc., Billerica, MA). Spectral analysis was done using the flexAnalysis v 3.4 software. Spectra were obtained using the direct transfer protocol supplied by the manufacturer. Colonies of each species were grown up overnight. Cells, including a bacterial test standard, were spread on a polished steel target plate and treated with formic acid before application of the matrix α-cyano-4-hydroxycinnamic acid (HCCA), 2 replicates per species. Measurements were made after the plate was dry. Spectra were collected using the flexControl v3.4 software over the 2 to 20 kilodalton range.

Spectra were obtained for strain ONC3 and compared to a database of reference spectra using the flexAnalysis software. The flexAnalysis software calculates a similarity score for the spectra, and the log of this score is used to estimate the strain similarity. Generally, a score greater than 2.3 is considered to be a match at the species level, 2 to 2.3 a match at the genus level, and 1.7 to 2 a probable genus level match Scores below 1.7 are regarded as unreliable. An absence of a respectable match when comparing OM1 to a reference database that contains type strains from the *Oxalobacteraceae* family (score of 2.87), could be indicative of a new genus under the family. However, the reference database is not fully comprehensive, so no conclusive new species can be made from this.

DNA Isolation, sequencing and genome annotation

To extract bacterial genomic DNA, the AllPrep Bacterial DNA/RNA/Protein kit (QIAGEN Inc., Germantown, MD) and the gDNA extraction from bacterial colonies protocol was used. The genomic library was prepared using the Illumina Nextera platform (San Diego, CA), and sequencing was performed using the NextSeq paired-end v2 Chemistry on v2.5 Flowcells at 150 bp per read, fragment length of 475 bp and target 20X coverage for reads.

After sequencing, a total of 10,528,120 reads were obtained at total read length of 1,552,795,738 bp. The assembly of the genome was carried out using SPAdes version 3.13.0 (Nurk et al., 2013). This produced 155 contigs, with an N_{50} value of 56,531 bp and a total assembled size of 5,622,601 bp. The G+C content of the genome was 63.82% and reads that met quality control standards were then mapped to contigs with an average genome coverage of 20X. The quality of the assembly was measure using BUSCO (Simao et al., 2015). The measure of completeness (40 single copy of BUSCO's) of 100%. Initial annotation of the genome was achieved using the PATRIC platform v 3.5.28 (Wattam et al., 2017). The Galaxy platform (Afgan et al., 2018) and RAST 2.0 (Aziz et al., 2008), were also used to annotate the genome and found that there were 5,104 protein coding sequences, 56 tRNA genes, and 2 rRNA operons.

16S rRNA phylogeny

Bacterial genomic DNA was extracted and prepared using the AllPrep Bacterial DNA/RNA/Protein kit (QIAGEN Inc., Germantown, MD) 16S *rRNA* sequences were

obtained from NCBI BLAST after blasting the 16S *rRNA* sequence and selecting the top 15 similar sequences. Sequences were downloaded as FASTA files and imported into MEGA 7 (Kumar et al., 2016) and aligned by MUSCLE (Edgar, 2004). A best-fit DNA model was then created with the aligned sequences and was implemented when running the phylogenetic analysis. Kimura-2-gamma with invariant sites (K2+G+I) (Jayaswal et al., 2007; Kimura, 1980) and bootstrapped 1,000 times with a partial deletion of 95% coverage cutoff were parameters that were set when creating the final phylogenetic tree (Figure 12). The tree was then visualized in MEGA 7 and iTOL (Letunic & Bork, 2016)

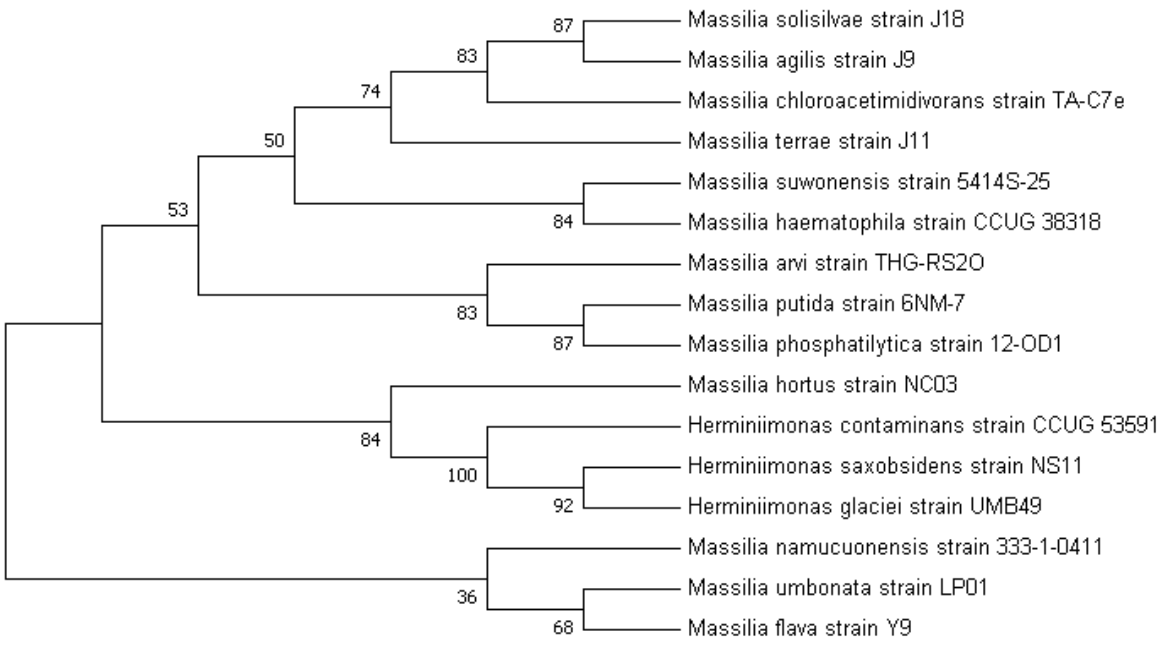

Figure 12. Maximum-likelihood phylogenetic tree based on the top similar 16S rRNA sequences from a BLAST search compared to ONC3. Values at branch points indicate bootstrap support as percentages based on 1000 resampling (only values greater than 50% are shown).

The maximum likelihood tree shows that *Massilia hortus* is most closely related to *Herminiimonas contaminans* strain CCUG 53591 which could indicate that these two

strains had a common ancestor and are related in that fashion. However, these strains do not share a common node, strain ONC3 is relegated to its own node whereas *H. cotaminans* strain CCUG 53591 shares a node with *H. saxobsidens* strains NS11. This is further confirmed with a bootstrap confidence value of 84 after 1000 bootstrap iterations. This also is reflected in similar findings of Kämpfer et. Al., 2013 (Kämpfer, P. Glaeser, Lodders, Busse, & Falsen, 2013) which shows that the *H*. *contaminas* strain CCUG 53591 is more closely related to other species of *Herminiimonas* and does have a common ancestor with strain NC03.

Genome Features

Sequencing and assembly of the genome of M. hortus strain ONC3 was performed by Novozymes North America (Durham, NC, USA) using the Illumina HiSeq 2500 system with paired end sequencing (2 x 150 bp). A total number of 10,528,120 reads with an average length of 147 bp (1,552,795,738 total bases) were obtained. Assembly quality was then accessed with BUSCO (Simao et al., 2015). The measured completeness (39 single copy BUSCO's, one duplicate BUSCO) was 97.5%. Genome assembly was performed by SPAdes version 3.13.0 (Nurk et al., 2013), which produced 155 contigs that had an N_{50} value of 55,531 bp and a total assembly size of 5,622,601 bp (5.62 MBp). The G+C content of the genome was 63.82%. Quality controlled reads were then mapped to contigs with an average genome coverage of 128-fold. Annotated contigs were then made using the PATRIC platform version 3.5.28 (Wattam et al., 2017) and the NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et al., 2016). This identified were 5,104 protein coding sequences, 56 *tRNA* genes, and 2 *rRNA* operons.

ONC3 can utilize L-histidine as a nitrogen source determined by the Biolog characterization. Histidine is regulated by the Hut pathway in bacteria (Bender, 2012). The *hutH* gene was found in the genome of ONC3 which is a part of the *HutP* regulon and encodes a histidase that aids in degradation of histidine into urocanate the final production of L-glutamate by catalyzation the nonoxidative elimination of the alpha-amino group of histidine (Schwede, Retey, & Schulz, 1999). The gene *hutU* was also found to be present in the genome as well and is also part of the *HutP* regulon. The *hutU* gene encodes for a urocanase that hydrates the urocanate into imidazlone propionate (Bender, 2012).

The MiGA platform (M. et al., 2018) in conjunction with the available Prokaryotic Pipeline was used to compare the *M. hortus* genome to other available genomes and found that *M. hortus* did belong to the genus *Massilia*, however it has a low affiliation with any other species in the genus. Using the Average Amino Identity (AAI) between similar genomes, it was found that the genome of *M. hortus* was 73.06% most closely related to Massilia putida NZ CP019038 (Table 14). The Genome-to-Genome-Distance Calculator was then utilized to calculate intergenomic distances and DNA-DNA hybridization (DDH) probabilities through digital analysis of 3 different models. When the genome of *M. hortus* was compared to that of 5 most similar genomes (*Massilia sp*. WG5, *Massilia putida* strain 6NM-7T, *Massilia oculi* strain CCUG 4327, *Massilia violaceinigra* strain B2, and *Massilia lutea* strain DSM 17473) it was found that the DDH probability values were lower than that of 70% for reference strains except for *Massilia sp*. WG5 which was 0.01% (Table 15). A threshold of <70% DDH similarity is typically

applied to justify the establishment of a new species (Alexander F. Auch et al., 2010). Based on these data, we propose the ONC3 is a novel species of the genus *Massilia* and should be classified taxonomically as *Massilia hortus* strain ONC3.

Table 14. Average amino acid index values of genomes like ONC3 when compared to each other.

Dataset	AAI (%)
Massilia putida NZ CP019038	73.06
Massilia sp. WG5 NZ CP012640	73.04
Janthinobacterium sp. LM6 NZ CP019510	65.42
Herbaspirillum seropedicae NZ CP011930	61.98
Collimonas fungivorans Ter331 NC 015856	61.91
Herminiimonas arsenitoxidans NZ LT671418	61.83
Pandoraea sputorum NZ CP010431	55.37
Paraburkholderia caribensis NZ CP013102	55.34
Paraburkholderia phytofirmans PsJN NC 010681	55.3
Burkholderia anthina NZ CM003768	55.29
Burkholderia sp. NRF60 BP8 NZ CP013373	55.25
Burkholderia mallei SAVP1 NC 008785	55.17
Cupriavidus taiwanensis NZ LT984801	55.08
Burkholderia insecticola NC 021287	54.83
Bordetella hinzii NZ CP012076	52.24

Table 15. GGDC output values using ONC3 as query genome and selecting 5 similar genomes using the largest genomic contig with 3 different best-fit models being used. ONC3 was used as the reference genome and was compared to 6 similar genomes based on the largest contig in the assembly. DNA-DNA hybridization values are denoted as DDH.

Query genome	Reference genome	DDH	Model C.I.	Distance	Prob. DDH >= 70%
ONC3	Massilia sp. WG5	25	[21.7 - 28.6%]	0.5978	0.01
ONC3	Massilia putida strain 6NM-7T	21.3	[18.1 - 24.9%]	0.6782	0
ONC3	Massilia oculi strain CCUG 4327	23.1	[19.8 - 26.7%]	0.6372	0
ONC3	Massilia violaceinigra strain B2	17.4	[14.4 - 21%]	0.7887	0
ONC3	Massilia lutea strain DSM 17473	18.5	[15.4 - 22.1%]	0.754	0
		Formula 2			
Query genome	Reference genome	DDH	Model C.I.	Distance	Prob. DDH >= 70%
ONC3	Massilia sp. WG5	22.6	[20.4 - 25.1%]	0.1935	0
ONC3	Massilia putida strain 6NM-7T	22.2	[19.9 - 24.7%]	0.1974	0
ONC3	Massilia oculi strain CCUG 4327	21.9	[19.6 - 24.3%]	0.2004	0
ONC3	Massilia violaceinigra strain B2	20.9	[18.7 - 23.4%]	0.2097	0
ONC3	Massilia lutea strain DSM 17473	21.2	[19 - 23.7%]	0.2068	0
Query genome	Reference genome	DDH	Model C.I.	Distance	Prob. DDH >= 70%
ONC3	Massilia sp. WG5	23.5	[20.6 - 26.6%]	0.6756	0
ONC3	Massilia putida strain 6NM-7T	20.5	[17.7 - 23.6%]	0.7417	0
ONC3	Massilia oculi strain CCUG 4327	21.9	[19.1 - 24.9%]	0.7099	0
ONC3	Massilia violaceinigra strain B2	17.2	[14.6 - 20.2%]	0.833	0
ONC3	Massilia lutea strain DSM 17473	18.1	[15.5 - 21.1%]	0.8049	0

Proposal Massilia hortus sp. nov, strain ONC3

Massilia hortus strain ONC3 can be distinguished from other type strains and members of the *Massilia* genus by chemotaxonomic characterization, genetic analysis including genome and 16S evolutionary phylogeny. Microlog chemotaxonomic testing showed that isolate ONC3 was most similar to *Massilia arenosa* strain MC02, which is also a novel strain of the genus Massilia. However, these strains differ in appearance as ONC3 is a light brown, circular, flat colony and not shiny whereas MC02 is a clear-yellow, shiny and semi-opaque colony that is slightly raised. 16S analysis using a phylogenetic tree also was used to confirm novelty of strain ONC3 as such that the ONC3 was contained to its own node with and its genomic average amino identity to a set of 5 similar genomes was also low at 73.06% provided by MiGA. The GGDC also produced results that provide evidence that strain ONC3 did not have similar genomic features that resemble other classified *Massilia* species. This suggest that *Candidatus Massilia hortus* nov. sp, strain ONC3 is a novel member of the genus *Massilia*.

Description of Massilia Hortus sp. nov., strain ONC3

M. hortus strain ONC3 was isolated from garden soil that had not been planted recently located near Maxton, NC, USA. Cells are gram negative rods in chain arrangements. Colonies were cultured on R2A media and are round, flat brown colored. Growth occurs at 21-30°C. Cells can utilize L-histidine and several sugars including maltose, trehalose, cellobiose and turanose. Cellular assimilation of L-histidine seems to be novel only to ONC3 whereas other *Massilia* that have been characterized do not

show assimilation of L-histidine. The genome of ONC3 is 5.62 Mbp and has a G+C content of 63.82

Culture Collection Deposit

Strain ONC3 was deposited into the American Type Culture Collection Depository and the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ).

Author Statements

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Ethical statement

The authors declare that the publication is not in consideration for any other journal, and that all authors agreed with its publication, and that no animal or human studies were carried out for this study.

Conflicts of interest

The authors declare that there are no conflicts of interest.

CHAPTER 5

Draft genome sequence of Duganella sp. DN04 isolated from cultivated soil

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Abstract

We sequenced *Duganella* sp. DN04, a novel species within the *Duganella* genus from a maize field in North Carolina. The assembled draft genome size is 6,562,230 bp, with a total of 6,039 protein coding sequences and 3,889 functionally assigned genes, including genes putatively involved in the colonization of plants.

Announcement

The Genus *Duganella* within the *Oxalobacteraceae* Family was identified in 1997 (Vikram et al., 2017) and consists of Gram negative, motile, aerobic, mesophilic bacteria that are mainly found in soils. Most *Duganella* strains produce the bis-indole pigment violacein which has antifungal and antibacterial properties (Maya et al., 2012; B. X. Zheng, Bi, et al., 2017). Other *Duganella* strains show proteolytic and lipolytic activities (Krishnamoorthy et al., 2016).

Duganella sp. DN04 was isolated from a maize field in North Carolina on June 9th, 2016 (geographical coordinates 36.1034, -78.4114, soil pH 5.8). Soil was added to phosphate-buffered saline, and dilutions were plated on R2A plates, incubated at 30°C for 1-2 days, followed by 20°C for 1-2 days. Repeated colony transfers and streaks were performed on

R2A to acquire a pure colony. Genomic DNA was extracted from a freshly grown R2A culture using the AllPrep Bacterial DNA/RNA/Protein kit (QIAGEN Inc., Germantown, MD) following the kit protocol. A genomic library was prepared using Illumina Nextera (San Diego, CA), size selected to an average fragment length of 475 bp and sequenced using Illumina NextSeq paired-end v2 Chemistry on v2.5 flowcells at 150 bp per read. A target coverage of 20X was used, and the genome was assembled using SPAdes 3.13.0 (Bankevich et al., 2012). Default parameters were used for all software unless otherwise specified. The genome was screened for possible contamination by blasting annotated coding regions that represented at least 60% of the contigs against a diverse set of genomes. If less than 10% of the hits were to proteins from other species in the same family, the contamination was considered to be low.

A total of 11,788,390 reads were obtained with a total read length of 1,738,895,421 bp, and an average read length of 148 bp. The genome length was 6,562,230 bp. In total, we found 281 contigs, with a N50 value of 40,161 (range: 671 to 141,855 bp) and an L50 value of 49. Assembly quality assessment using BUSCO (Simao et al., 2015) revealed a measured completeness (40 single copy BUSCO's) of 100%. Following genome annotation by PATRIC 3.5.28 (Wattam et al., 2017), we identified a total of 6,039 protein coding sequences with 2,150 hypothetical genes, and 3,889 genes with functional assignments. DN04 has 48 tRNA genes, 4 rRNA genes, 43 antibiotic resistance genes, and a GC content of 64.4%. Based on 16S rRNA, *Duganella sacchari* strain Sac-22 is the closest related species (98.82% identity).

Galaxy and RAST 2.0 were used to annotate and identify specific genes (Afgan et al., 2018; Overbeek et al., 2005). We discovered several genes that are putatively involved in plant-growth promotion, including genes needed to catalyze the decomposition of hydrogen peroxide (*katE*), and genes involved in the production of urease (*ureA-G*), biofilms (*bdcA, and wspC*) (Barraud, Kjelleberg, & Rice, 2015), and biotin (*bioA-D, bioF*) (Streit, Joseph, & Phillips, 1996), and several genes of the Pst operon and a two-component signal transduction system involved in phosphate uptake (*phoR, phoB, phoD, pstS,C,A,B, phoU, ppk*) (Asaf, Khan, Khan, Al-Harrasi, & Lee, 2018). We also identified 60 putative virulence genes, including 45 antibiotic and toxic compound resistance genes and 15 invasion and intracellular resistance genes.

Data availability

The complete genome sequence has been deposited in NCBI/GenBank under BioProject number PRJNA529278, BioSample number SAMN11263562, accession number SPVG00000000, and SRA accession number SRX6098754.

Acknowledgements

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Duganella callidus sp. nov., a novel addition to the Duganella Genus, isolated from the soil of a cultivated maize field

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Correspondence: Heike Bücking, heike.bucking@sdstate.edu
Keywords: *Duganella, Oxalobacteraceae*
Abbreviations:
Repositories: The genome has been deposited at DDBJ/EMBL/GenBank under the BioProject number PRJNA529278 (https://www.ncbi.nlm.nih.gov/bioproject/529278), BioSample number SAMN11263562, and Assembly GCA004614165.
Status: close to submission to the journal International *Journal of Systematic and Evolutionary Microbiology*. Papers are ready and waiting on paperwork for the culture collection banks.

Abstract

A gram negative, rod-shaped bacterium, strain DN04, was isolated from a maize field soil sample, collected from North Carolina, USA. The most similar *Duganella* species based on the 16S rRNA are *Duganella sacchari* Sac-22, *Duganella ginsengisoli* DCY83, and *Duganella radicis* Sac-41 (98.82, 97.57, 97.49 percent identity respectively). Based on Biolog GenIII phenotypic testing, DN04 is unique in several carbon and amino acid utilization parameters such compared to three of the closest isolates; D-Turanose, N-Acetyl-D-Glucosamine, Inosine, and L-Pyroglutamic acid. DN04 had low similarity to any other isolate in the MALDI-TOF database, with 1.44 being the most similar score which is much below the 1.7 threshold for a new isolate. Full genome comparison tools were used to help determine if DN04 is a novel species. The most similar AAI (average amino acid identity) score was 68.93%. and the most similar ANI (average nucleotide identity) score was 83.79% which indicates DN04 is a new species. The genome-to-genome-distance calculation (GGDC) also showed DN04 to be a new species, due to the 0.03 DDH probability of being < 70%, which is the new species threshold. Based on the

morphological, phenotypic, and genomic differences, DN04 appeared to be a novel species of the *Duganella* genus, with the proposed name of *Duganella callidus* sp. nov. DN04.

Introduction

As our non-culture dependent identification methods and sequencing technologies improve, additional beneficial soil microorganisms are being taxonomically identified (Hirsch, Mauchline, & Clark, 2010; Su, Lei, Duan, Zhang, & Yang, 2012). It is important to expand our knowledge of the microbes that colonize the rhizosphere and phyllosphere, so we are able to have a better understanding of how they can be utilized to improve plant and soil health (Wozniak & Galazka, 2019).

Oxalobacteraceae is a family of bacteria in the Burkholderiales order, Betaproteobacteria class, and in the Proteobacteria phylum (Garrity et al., 2015). *Oxalobacteraceae* are gram negative, non-spore forming, heterotrophic, and the majority are mesophilic and aerobic/microaerobic (Garrity, 2005). The varying morphological forms are rods, curved rods, vibrio or spirillum like. *Oxalobacteraceae* is a very metabolically, ecologically, and phenotypic diverse group of bacteria (Jose Ivo Baldani et al., 2014).

The first member of the *Duganella* genus was isolated from waste water in 1968, and first classified as *Zoogloea ramigera* (Friedman & Dugan, 1968). However in 1997, after 16S rRNA sequencing and additional phenotypic and phylogenetic analysis, *Zoogloea ramigera* was reclassified as a new genus in the *Oxalobacteraceae* family

(Hiraishi A, 1997). The new genus was named *Duganella* after Dugan the original microbiologist who isolated the organism (Hiraishi A, 1997). Since the initial formation of the *Duganella* genus in 1997, five additional species were added to the genus. *D. violaceinigra* was isolated from forest soil (W. J. Li et al., 2004), *D. phyllosphaerae* from a *Trifolium repens* leaf surface (Kampfer P, 2012), *D. sacchari* and *D. radicis* were both isolated from the rhizosphere of sugar cane (Madhaiyan et al., 2013), and *D*. *ginsengisoli* was isolated from ginseng soil (J. Zhang et al., 2016).

Duganella are gram negative, straight or curved rods, aerobic, motile, non-spore forming, mesophilic bacteria (Hiraishi, Shin, & Sugiyama, 2015). *Duganella* have been found to have close genetic similarities with bacteria from the genera *Massilia, Janithobacterium*, *Pseudoduganella*, and *Telluria* (Jose Ivo Baldani et al., 2014; Garrity, 2005; Valdes et al., 2015). *Duganella* contributes to the biogeochemical cycling of toxic selenite to non-toxic forms in soils (Bajaj, Schmidt, & Winter, 2012). Most *Duganella* species produce violacein, a bisindole. Violacein has been of interest due to the research indicating its antibacterial (S. Y. Choi, Kim, Lyuck, Kim, & Mitchell, 2015), antiviral (Andrighetti-Frohner, Antonio, Creczynski-Pasa, Barardi, & Simoes, 2003), anti-protozoan(Matz et al., 2004), and anti-cancer (Hashimi, Xu, & Wei, 2015) properties. *Duganella* species have also been shown to solubilize phosphorus, potassium, and zinc (Verma, Yadav, Kazy, Saxena, & Suman, 2014). Several strains of *Duganella* were shown to have antifungal capabilities against the plant pathogen *Fusarium graminearum*, due to amylolytic, lipolytic, and some chitinoclastic activity (Haack et al., 2016).

Isolation

Duganella callidus DN04 was isolated from a maize field in North Carolina on June 9th, 2016. The soil pH was 5.8 and the geographical coordinates of the isolation were 36.1034, -78.4114. The bacterium was cultured by adding soil to phosphate-buffered saline, and plating dilutions on R2A medium, then incubating at 30°C for 1-2 days, followed by 20°C for 1-2 days. Colonies were transferred to new R2A plates to acquire a pure colony. The pure colonies were transferred to R2A broth and grown at 30°C for 24 h and transferred to 20% glycerol solution for storage at 80°C for future testing.

Morphology

Duganella callidus was grown on R2A at 25°C for 3 days, and then form, elevation, and margin of individual colonies was determined, and a Gram strain was performed (Willgohs & Bleakley, 2009). Colonies of DN04 have a circular form, the elevation is flat, and the margins are entire, they are medium in size, yellow in colored and opaque in appearance. The bacteria are gram negative rods that are arranged in singles and doubles (Figure 13). Based on the 16S rRNA BLAST results, the two most similar bacterial strains, available in culture collections, were selected for morphological comparisons to DN04 (Figure 14A). In contrast, the colonies of *Massilia albidiflava* 45 have a diameter of 1.0-1.5 mm and are circular, convex with entire margins, desiccated texture, opaque and pale yellow in color (Y. Q. Zhang et al., 2006) (Figure14B). *Duganella zoogloeoides* IAM 12670 colonies have a viscous appearance, desiccated

texture, convex with entire margins, are slightly transparent, and cream colored (Hiraishi A, 1997) (Figure14C). Compared to these reference strains, the colonies of DN04 were more opaque and yellow in appearance and were more creamy textured, compared to the viscous pale yellow and desiccated structure of *M. albidiflava* and *D. zoogloeoids* colonies.

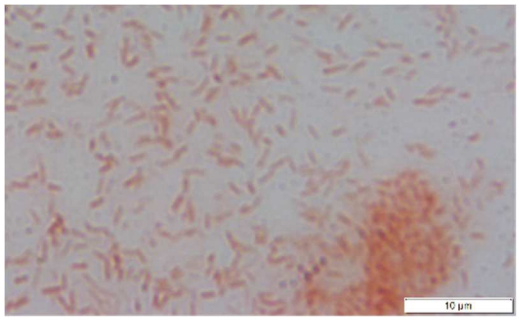

Figure 13. Gram-strain of DN04, 10µm scale.

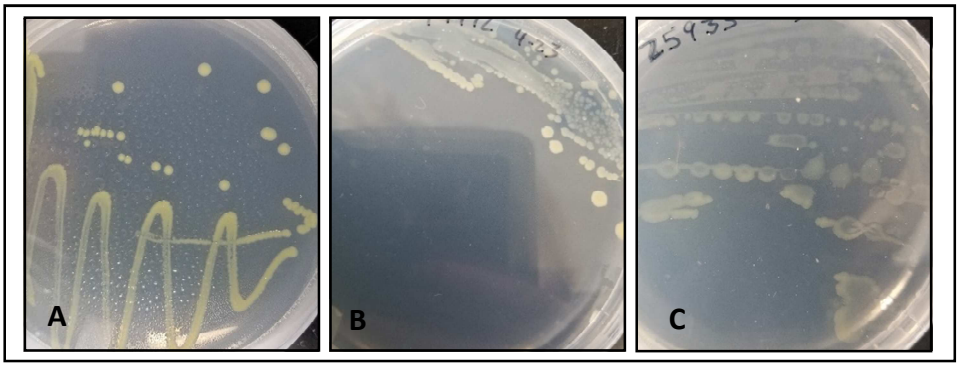

Figure 14. Colony morphology on R2A media grown at 30 °C for 3 days. A) *Duganella callidus* DN04, B) *Massilia albidiflava* 45, C) *Duganella zoogloeoides* IAM 12670

16S RNA phylogeny

DN04 genomic DNA was extracted from a freshly grown R2A broth culture using the AllPrep Bacterial DNA/RNA/Protein kit (QIAGEN Inc., Germantown, MD) following the kit protocol. Illumina Nextera (San Diego, CA) was used to prepare the genomic library, with an average selected size of about 475 bp in fragment length. They were

sequenced using the (Illumina) NextSeq paired-end v2 Chemistry on v2.5 Flowcells at 150bp per read. Default parameters were used for all software unless otherwise specified. Samples were sequenced to target 20x coverage, and the genome was assembled using Spades 3.13.0 (Bankevich et al., 2012). Assembly quality assessment using BUSCO (Simao et al., 2015) revealed a measured completeness (40 single copy BUSCO's) of 100%. PATRIC (Wattam et al., 2017) was used for annotation to determine genomic information, and the Galaxy platform (Afgan et al., 2018) was used for individual gene annotation.

Once sequenced, assembled, and annotated the 16S rRNA contig was used to identify the closest related sequences from the NCBI Basic Local Alignment Search Tool (BLAST) nucleotide database (Zhang Z., 2000). The most similar sequences as well as the most similar additional genera from the *Oxalobacteraceae* family, and *Burkholderia metallica* R-16017 to root the phylogenetic tree, were downloaded as FASTA files and MEGA 7 was used to create a phylogenetic tree (Kumar et al., 2016). To align the sequences MUSCEL was used with the default settings applied (Edgar, 2004). A phylogenetic tree was created using the Maximum Likelihood statistical method, with 1000 bootstrap replications, and partial deletion with a 95% coverage cut-off. Based on the 16S rRNA tree *Duganella sacchari* strain Sac-22 and *D. callidus* DN04 share the most similar common ancestor, and *Duganella ginsengisoli* strain DCY83 is the second closest related strains (Figure 15). When conducting a BLAST on the 16S rRNA, *D. callidus* had a 98.82% identity with *D. sacchari* Sac-22, and a 97.57% identity with *D. ginsengisoli* DCY83. The tree also shows that *Duganella callidus* forms its own distinct branch.

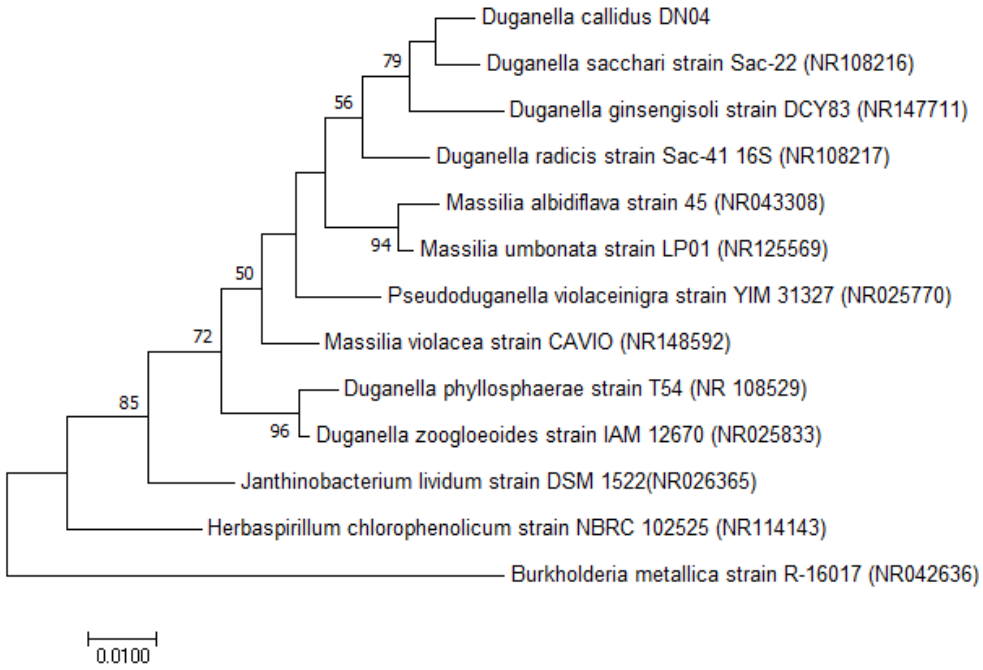

Figure 15. Maximum-likelihood phylogenetic tree created using MEGA 7 based on the most similar 16S rRNA gene sequence analysis using DN04 as the query sequence. The numbers at the nodes indicate bootstrap percentages (based on 1000 replications). Bootstrap values above 50% are shown at the nods. *Burkholderia metallica* R-16017 is utilized to root the tree.

Physiology and Chemotaxonomy

The Biolog Gen III MicroPlate test system (Biolog, Hayward, CA, USA) was used to determine the chemotaxonomic phenotype. This 96 well plate system contains a positive and negative control, 23 chemical sensitivity assays, and 71 carbon source utilization assays. All the cultures were tested in accordance with the Biolog Gen III manufacturer manual (BIOLOG, 2013). Plates were inoculated with the appropriate inoculation fluid (Supplementary Table 21), and incubated for 120 h at 30°C. The plates were read using the Biolog's Microbial Identification Systems software (e.g.OmniLog® Data Collection) and analyzed.

Duganella callidus DN04 was phenotypically characterized and compared to additional isolates from the *Oxalobacteraceae* family. Fifteen additional isolates were selected based on their 16S rRNA sequence similarity and availability in culture collection banks. The fifteen isolates represented a broad spectrum of genera within the *Oxalobacteraceae* family. In total, we compared DN04 to one strain of the genus, *Collimonas, Herminiimonas, Janthinobacterium, Duganella, Undibacterium, Oxalicibacterium, Telluria, Glaciimonas, Herbaspirillum*, and *Noviherbaspirillum*, and five different *Massilia* strains. The cultures were acquired from the American Type Culture Collection (ATCC, Manassas, USA), DSMZ (Braunscheig, Germany) or the VTT Technical Research Centre of Finland (VTT, Finland). The selected isolates are shown in (supplementary material) Table 20, with the corresponding accession number and supplier information.

Figure 16 represents a nonmetric multi-dimensional scaling (MDS) plot was created to visualize the Biolog results. The “+”, “-”, “+/-” results were converted into the numerical form 2, 0, 1, respectively. R-studio was used to create the nonmetric MDS plot (Ross Ihaka & Robert Gentleman, 1996). According to this analysis DN04 is most closely related to *Duganella zoogloeoides* IAM 12670, *Massilia dura* 16, and *Massilia albidiflava* 45. Based on the Biolog results we can conclude that *Duganella callidus* DN04 is phenotypically most similar to the genus *Duganella* and *Massilia.* However, its phenotypic characteristics sets it apart from these bacterial species.

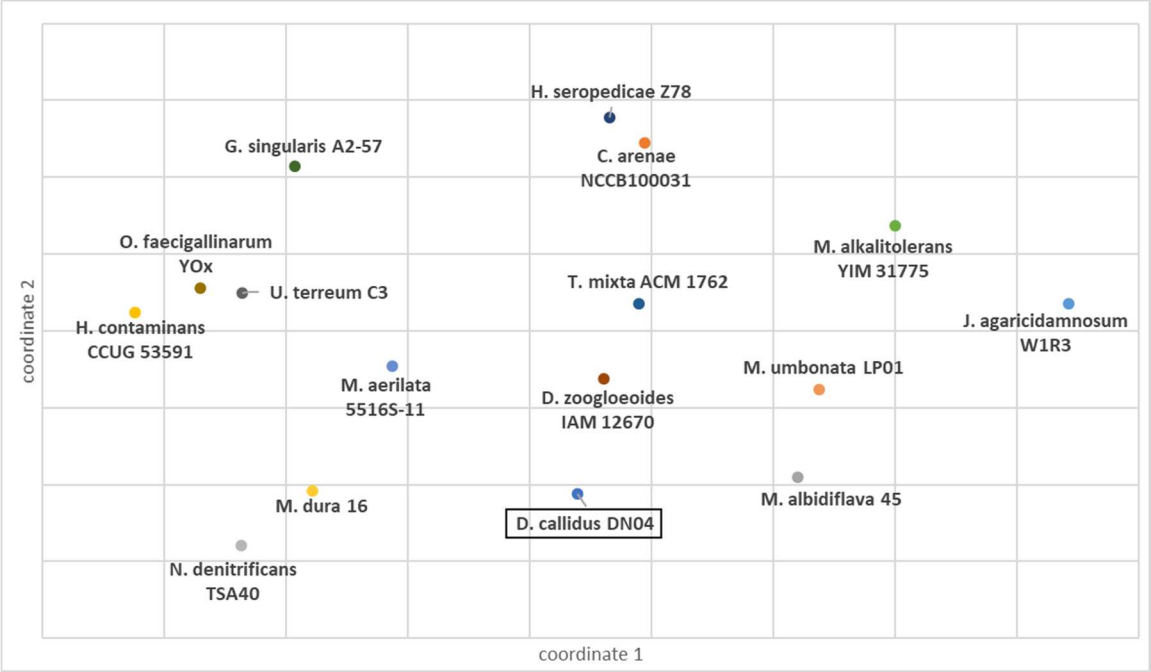

Figure 16. Non-metric multidimensional scaling plot based on the Biolog results of DN04 and similar genera from a 16S rRNA BLAST and availability in culture collections. Isolate information in supplemental Table 20.

Table 16 further identifies some of the specific differences between *D. callidus* and three of the most similar isolates from the Biolog results: *Duganella zoogloeoides* IAM 12670, *Massilia dura* 16, and *Massilia albidiflava* 45. Table 16 shows that *D. callidus* was able to utilize sources such as D-turanose, N-acetyl-D-glucosamine, inosine, and L-pyroglutamic acid that two or all three of the most similar isolates were unable to metabolize. N-acetyl-D-glucosamine degradation through N-acetyl-D-glucosamine kinase (*nagK*) has been found to play a role in cell wall murein recycling (Uehara & Park, 2004), and we identified in the genome of DN04 the *nagK*_1-3 genes. The genome of DN04 shows also putative inosine-5'-monophosphate dehydrogenase (*guaB*_1-4) genes which plays a role in inosine utilization. Inosine phosphate is converted to xanthosine monophosphate by the *guaB* enzyme, which is essential for the purine nucleotide biosynthetic pathway (Leyssen, Charlier, Paeshuyse, Clercq, & Neyts, 2003). DN04 also

possesses the glutathione-specific gamma-glutamylcyclotransferase gene (*chaC*) which is one component of the glutathione metabolization pathway. The protein is involved in the synthesis and break down of glutathione with L-pyroglutamic as an intermediate component (Niehaus, Elbadawi-Sidhu, de Crecy-Lagard, Fiehn, & Hanson, 2017). In contrast, *D. callidus* was unable to utilize several carbon and amino acid sources that two or all three of the reference strains were able to utilize, such as L-fucose, glycerol, L-alanine, pectin, and α-keto-glutaric acid (Table 16). In addition to these chemotaxonomic differences, DN04 differed from the reference strains in its sensitivity testing. DN04 was not able to grow in the presence of the oxidant tetrazolium blue, and the antibiotics lincomycin and aztreonam (Table 16).

Table 16. Differing Biolog results between *Duganella callidus* DN04, and *Duganella zoogloeoids* IAM 12670, *Massilia albidiflava* 45, *Massilia umbonata* LP01. All plates were incubated for four days, and the proper growth medium and inoculation fluid is indicated in the supplemental Table 21.

Biochemical Test	***Duganella callidus***	***Duganella zoogloeoids***	***Massilia albidiflava***	***Massilia umbonata***
Description	DN04	ATCC 25935	DSMZ 17472	DSMZ 26121
Utilization of:				
D-Turanose	+	-	-	-
N-Acetyl-D-Glucosamine	+	-	+	-
L-Fucose	-	±	+	+
Inosine	+	+	-	-
Glycerol	-	+	-	+
L-Alanine	-	+	+	+
L-Pyroglutamic Acid	+	-	+	-
Pectin	-	+	+	+
α-Keto-Glutaric Acid	-	±	+	+
Resistance to:				
Tetrazolium Blue	-	±	+	+
Linocomycin	-	+	+	+
Aztreonam	-	+	+	+

MALDI-TOF

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a tool utilized to identify bacteria based on isolates spectra and comparing it to a database using bioinformatics pattern profiling (Singhal et al., 2015). The MALDI-TOF MS analysis is carried out on the Bruker microflex instrument, utilizing the flexControl v3.4 (Bruker Daltonics Inc., Billerica, MA). Spectra were attained using the direct transfer protocol indicated by the manufacturer, and FlexAnalysis v3.4 software was used for the spectral analysis. DN04 cells were preparted for analysis by growing the colonies overnight, then the cells as well as a bacterial test standard were spread on a polished steel target plate and treated with formic acid. They were then applied to the matrix which is made up of α-Cyano-4 hydroxycinnamic acid (HCCA). After the plates were dry, the spectra were collected and analysied using the flexControl v3.4 software over the 2 to 20 kiloDalton range. Two replications were conducted on DN04.

Once the spectra were obtained it can be compared to a reference spectra database using the flexAnalysis software. The strain similarity score is calculated based on the log of the spectra score. The scores help indicate how similar DN04 is to other strains in the database, as well as if it is a novel isolate. Generally, a score of greater than 2.0 is considered to be from the same species, and a score of 1.7 to 2.0 is considered to be a match at the genus level. Scores lower than 1.7 suggest there are no similar bacterial spectra in the database, and the bacteria in question is suggested to be a novel bacterium. When DN04 was analyzed, it was compared against itself as a control, as well as the MALDI-TOF database. The results indicate that *D. calliuds* is most

likely a novel isolate based on the highest MALDI-TOF score being 1.44 which is below the threshold for a new isolate.

Genome Features

Genomic DNA was sequenced as previously described using Illumina NextSeq and assembled using SPAdes 3.11.0 (Bankevich et al., 2012). Initial genome annotation was performed with the PATRIC platform and confirmed with RAST (Overbeek et al., 2005; Wattam et al., 2017). According to this pipeline, the genome length of *Duganella callidus* DN04 was 6,562,230 bp. The sequencing data resulted in a total of 11,788,390 reads, with a total read length of 1,738,895,421 bp, and an average length of 148 bp. This produced 281 contigs, with a N50 value of 40,161 (range: 671 to 141,855 bp), a L50 value of 49, and a GC content of 64.4%. After annotation we identified a total of 6,039 protein coding sequences with 2,150 hypothetical genes, and 3,889 genes that could be functionally assigned. In the genome of DN04 48 tRNA genes, 4 rRNA genes, and 43 antibiotic resistance genes were identified.

The Galaxy platform was used to annotate and identify individual genes (Afgan et al., 2018). DN04 has several putative plant-growth promoting genes including genes used to catalyze the decomposition of hydrogen peroxide (k*atE*), urease biosynthesis (*ureA*-*G*), biofilm production (*bdcA*, and *wspC*), biotin synthesis (b*ioA*-*D*, b*ioF*), and several phosphorus regulatory genes (*phoR*, p*hoB*, p*hoD*, p*stS,C,A,B*, p*hoU*, p*pK*). Using RAST 2.0 (Overbeek et al., 2005), we also identified 60 putative virulence genes, including 45 antibiotic and toxic compound resistance genes. DN04 has 15 invasion and intracellular

resistance genes, and 109 flagella motility genes. Due to the flagella genes and the invasion/intracellular resistance genes, *Duganella callidus* is a potential plant endophyte (Czaban et al., 2007).

We used the prokaryotic pipeline of MiGA to determine taxonomic ranking and novelty (M. et al., 2018). MiGA uses average amino acid identity (AAI) to determine the closest related strain in the NCBI database. Based on AAI, DN04 is most similar to *Janthinobacterium* sp. 1 2014 MBL MicDiv NZ CP011319 and *Janthinobacterium* sp. LM6 NZ CP019510 with an AAI of 68.93% and 68.82% respectively. This is similar to additional whole genome comparisons in literature, where the analysis revealed a novel strain of *Janthinobacterium* was most similar to a member of the genus *Duganella (Valdes et al., 2015)*. These AAI values are just above the novel genus cutoff value of 65%. Since *Janthinobacterium* sp. 1 2014 MBL MicDiv NZ CP011319 had the highest AAI score it was used as the reference sequence for the p-value comparisons in Table 17. The taxonomic novelty uses p-values to indicate the probability of getting an AAI less than or equal to the query AAI value. The analysis demonstrates that DN04 belongs to the *Oxalobacteraceae* family, and that *Duganella callidus* DN04 is a novel species (p-value of 0.00924 for species) (Table 17).

Table 17. Taxonomic classification and novelty based on p-values of sequence AAI comparison using the MiGA tool with *Duganella callidus* DN04 as the query and *Janthinobacterium* sp. 1 2014 MBL MicDiv NZ CP011319 as the reference sequence.

Taxonym	Taxonomic classification p-value	Taxonomic novelty p-value
Order	0.000	0.875
Family	0.000	0.739
Genus	0.111	0.519
Species	0.374	0.00924
Subspecies	0.422	0

Average Nucleotide Identity by Orthology (OrthoANI) utilizes the original Average Nucleotide Identity (ANI) algorithm but expands it to also evaluate the reciprocal best hits. Once the genomes of interest are divided into 1020 bp-long fragments and a search is conducted using NCBI BLASTn the two sequences are compared. The OrthoANI identifies the reciprocal best hits (orthologous relationship), by running reciprocal BLASTn searches with each fragment (Lee et al., 2016). OrthoANI is used to compare the two sequences using EzBioCloud (Yoon, Ha, Lim, et al., 2017). The cutoff boundary for a new bacteria species using OrthoANI on EzBioCloud is ~95-96% (Goris et al., 2007). The OrthoANI values of the six closed related species to DN04 using NCBI BLASTn are shown in Table 18. All six of the closest related species have an OrthoANI value lower than the 95-96% novel species cutoff value. The two highest OrthoANI values correspond to *Duganella sacchari* Sac22 and *Duganella* sp. HH101 with values of 83.79% and 82.92% respectively. Based on these results DN04 most likely belongs to the *Duganella* genus but is a novel species within this genus.

Table 18. OrthoANI values from EZBioCloud of six genetically similar strains compared to *Duganella callidus* DN04.

Isolate	OrthoANIu value (%)
Duganella sacchari Sac22	83.79
Duganella sp. HH101	82.92
Massilia sp. NR4 -1	78.73
Massilia armeniaca ZMN-3	77.94
Janthinobacterium sp. 1-2014MBL MicDiv	77.22
Massilia violaceinigra B2	76.94

This is also confirmed by genome-to-genome distance-calculations (GGDC) which is able to measure intergenomic distance, much like the traditional wet-lab DNA-DNA hybridization (DDH) values (Meier-Kolthoff et al., 2013). GGDC was calculated based on

the intergenomic distance between *D. callidus* and eight of the most similar full genomes based on a NCBI BLAST using the 16S rRNA contig. GGDC produces three different formulas for calculating intergenomic distances, and formula 2 is shown in Table 19. Formula 2 is calculated by taking the sum of all identities found in high-scoring segment pairs (HSPs) divided by overall HSP length, and it is the most robust formulation because it is independent of genome length (A. F. Auch et al., 2010). Table 19 results show that all 8 of the genomes had a DDH probability below the 70% threshold which predicts DN04 is a new species. This is also enforced by the model confidence intervals, as well as the G+C differences that are close to or greater than 1 for most of the reference genomes (Meier-Kolthoff, Klenk, & Goker, 2014). The closest related genomes to DN04 based on the GGDC distance calculation from formula 2 are: *Duganella sacchari* Sac-22, *Duganella phyllosphaerae* T54 DUPY, and *Duganella zoogloeoides* ATCC 25935.

Table 19. GGDC formula 2 values based on DN04 as the query, compared to the top eight most similar full genomes based on BLAST results from the 16S rRNA contig.

Formula 2: Sum of all identities found in HSPs divided by overall HSP length						
Query genome	**Reference genome**	**DDH**	**Model C.I.**	**Distance**	**Prob. DDH >= 70%**	**G+C difference**
Duganella callidus DN04	*Duganella sacchari* Sac-22 (GCA_900143065)	27.1	[24.8 - 29.6%]	0.1593	0.03	2.74
Duganella callidus DN04	*Duganella phyllosphaerae* T54 DUPY (GCA_001758785)	23.9	[21.6 - 26.4%]	0.1828	0	1.19
Duganella callidus DN04	*Duganella zoogloeoides* ATCC 25935 (GCA_000383895)	23.1	[20.8 - 25.5%]	0.1895	0	0.3
Duganella callidus DN04	*Massilia albidiflava* DSM 17472 (GCA_004322755)	21.5	[19.3 - 23.9%]	0.2041	0	1.36
Duganella callidus DN04	*Janthinobacterium* sp.1-2014MBL MicDiv (GCA_001865675)	21.4	[19.2 - 23.9%]	0.2048	0	0.79
Duganella callidus DN04	*Massilia umbonata* DSMZ 26121 (GCA_005280315)	21.4	[19.1 - 23.8%]	0.2056	0	0.9
Duganella callidus DN04	*Janthinobacterium agaricidamnosum* BHSEK (GCA_003667705)	21.3	[19.1 - 23.8%]	0.2059	0	1.54
Duganella callidus DN04	*Massilia violaceinigra* B2 (GCA_002752675)	20.9	[18.7 - 23.3%]	0.2102	0	0.91

Proposal of Duganella Callidus sp. nov. DN04

Due to the morphological, phenotypic, and genomic differences that were identified, we conclude that DN04 is a novel species of the *Duganella* genus. DN04 has

different color and texture of colonies on R2A media than two similar isolates based on 16S rRNA, *D. zoogloeoidis*, and *Massilia albidiflava.* The Biolog Gen III results indicate the chemotaxonomic phenotype of DN04 is most similar to *D*. *zoogloeoidis, M. albidiflava, and M. umbonata.* However, DN04 has distinct phenotypic differences from these reference isolates (Table 16), and many of these differences are supported by the annotation of the genome. The phylogenetic tree shows that DN04 is most closely related to *D. sacchari* Sac-22 but forms its own branch. Based on the MALDI-TOF results*, D. callidus* is a novel isolate, due to there being no similar spectra in the database. The highest score that was obtained was 1.44 which is below the threshold for a novel isolate. The AAI taxonomic novelty p-value of 0.00924 also indicates that DN04 is distinctly different on a species level from other bacterial genomes in the NCBI database. According to the OrthoANI calculation, the closest related genome sequence is *D. sacchari* Sac22, but the OrthoANI value of 83.79% is much below the novel species cutoff value of ~95-96%, indicating DN04 is most likely in the *Duganella* genus but a novel species. This finding is also confirmed by the GGDC values, when comparing the genome of DN04 to *D. sacchari* Sac22, the DDH value is 27.1, distance value is 0.1593, GC difference above 1, and a 0.03% probability that the two genomes would have a DDH below the new species threshold. These analyses indicate that DN04 is a novel species in the *Duganella* genus.

Description of Duganella Callidus sp. nov. DN04

Duganella callidus sp. nov. [kal.li.dus adj. meaning clever/cunning, as initially it was not depicted by a single genus but rather several, until in depth analyses were run to determine it was a *Duganella* genus]

Duganella callidus DN04 was isolated from a maize field in North Carolina, U.S.A. Its colony morphology is circular, flat, margins are entire, medium in size, yellow colored and opaque in appearance. Cells are gram negative rods in singles and doubles. It grows on R2A media, with a temperature range of 21-30°C. Based on the Biolog results, it can grow at a pH of 6 but it cannot grow at a pH of 5 or at a salt concentration of 1% or higher. DN04 metabolizes sugars such as; Dextrin, D-Maltose, D-Trehalose, D-Cellobiose, Gentiobiose, D-Turanose, a-D-Lactose, D-Salicin, N-Acetyl-D-Glucosamine, N-Acetyl-D-Galactosamine, a-D-glucose, D-Mannose, D- Fructose, D-Galactose, L-Rhamnose, and Inosine. *D. callidus* can assimilate various amino acids such as; Gelatin, Glycyl-L-Proline, L-Glutamic Acid, L-Histidine, and L-Pryoglutamic Acid. DN04 does not seem to be resistant to many antibiotics. *D. callidus* has sever putative endophytic and plant-growth promoting genes; (*katE*), (*ureA-G*), (*bdcA*, and *wspC*), (p*hoR*, p*hoB*, p*hoD*, p*stS,C,A,B*, p*hoU*, p*pK*), as well as 60 putative virulence genes, 45 antibiotic and toxic compound resistance genes, 15 invasion and intracellular resistance genes, and 109 flagella motility genes. The genome is 6.56 Mbp and GC content is 64.4%.

Table 20. Information on cultures purchased for Biolog Gen III comparison.

Isolate	Accession number	Culture Collection
Collimonas arenae strain NCCB 100031	NR_042824.1	DSMZ 21398
Herminiimonas contaminans strain CCUG 53591	NR_108871.1	DSMZ 28178
Janthinobacterium agaricidamnosum strain W1r3	NR_026364.1	DSMZ 9628
Duganella zoogloeoides strain IAM 12670	NR_025833.1	ATCC 25935
Undibacterium terreum strain C3	NR_109599.1	DSMZ 102222
Oxalicibacterium faecigallinarum strain YOx	NR_112834.1	DSMZ 21641
Telluria mixta strain ACM 1762	NR_044833.1	ATCC 49107
Glaciimonas singularis strain A2-57	NR_109670.1	DSMZ 100199
Massilia aerilata strain 5516S-11	NR_044355.1	DSMZ 19289
Massilia umbonata strain LP01	NR_125569.1	DSMZ 26121
Noviherbaspirillum denitrificans TSA40	NR_157007.1	ATCC TSD-69
Massilia dura strain 16	NR_043307.1	DSMZ 17513
Massilia alkalitolerans YIM 31775	NR_0430941	VTT 032361
Herbaspirillum seropedicae Z 78	AJ238361	DSMZ 6446
Massilia albidiflava 45	AY965999	DSMZ 17472

Table 21. Biolog results for *Duganella callidus* DN04 and all comparable species from supplemental Table 16. Plates were incubated for four days and the growth medium and inoculation fluid is identified on the table.

Biochemical Test	*Duganella Callidus*	*Massilia albidiflava*	*Collimonas arenae*	*Herminiimonas contaminans*	*Inhtinobacterium agaricidamnosum*	*Naxibacter (Massilia) alkalitolerans*	*Herbaspirillum serpedicae*	*Duganella zoogloeoids*	*Undibacterium terricum*	*Oxalicibacterium flocculinarum*	*Telluria mixta*	*Glacimonas singularis*	*Massilia aerilata*	*Massilia umbonata*	*Noviherbaspirillum denitrificans*	*Massilia dura*
Reactions	DN04	DSMZ 17472	DSMZ 21398	DSMZ 28178	DSMZ 9628	VTT 032361	DSMZ 6446	ATCC 25935	DSMZ 102222	DSMZ 21641	ATCC 49107	DSMZ 100199	DSMZ 19289	DSMZ 26121	ATCC TSD-69	DSMZ 17513
Inoculation Fluid	B	A	A	A	A	A	B	A	B	A	B	A	A	A	B	B
Media	R2A	R2A	Nutrient	TSA	Nutrient	TSB	Nutrient	TSA	R2A	Nutrient	Nutrient	R2A	R2A	R2A	R2A	TSA
Negative Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dextrin	+	+	-	-	+	±	±	+	-	-	+	-	+	+	+	+
D-Maltose	+	+	-	-	+	±	-	+	-	-	+	-	+	+	+	+
D-Trehalose	+	+	-	+	+	+	-	+	-	-	+	-	-	+	+	+
D-Cellobiose	+	+	-	-	+	+	-	+	-	-	±	-	+	+	-	+
Gentiobiose	+	+	-	+	+	+	-	+	-	+	-	-	+	+	-	+
Sucrose	-	+	-	-	+	+	-	-	-	-	-	-	-	+	+	+
D-Turanose	+	-	-	-	+	±	-	-	-	-	-	-	±	-	+	+
Stachyose	-	-	-	-	+	+	-	-	-	-	-	-	-	+	+	-
Positive Control	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH 6	+	+	+	-	+	+	+	+	-	±	+	+	+	+	+	+
pH 5	-	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-
D-raffinose	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-	-
a-D-Lactose	+	+	-	±	+	-	-	-	-	-	-	-	-	±	+	-
D-Melibiose	-	+	-	-	+	±	-	-	-	-	-	-	-	+	+	-
B-Methyl-D-Glucoside	-	+	-	-	+	+	-	-	-	-	+	-	-	-	-	-
D-Salicin	+	+	-	-	+	+	-	+	-	-	+	-	-	±	-	-
N-Acetyl-D-Glucosamine	+	+	+	-	+	+	±	-	-	-	+	+	-	-	+	-
N-Acetyl-B-D-Manosamine	-	-	-	-	+	-	-	-	-	-	±	-	-	-	+	-
N-Acetyl-D- Galactosamine	+	+	-	-	+	-	-	-	-	-	±	-	-	+	-	-
N-Acetyl-D- Galactosamine	-	-	-	-	+	-	-	-	-	-	±	-	-	-	-	-
1% NaCl	-	-	-	-	+	+	+	+	-	+	+	-	-	-	+	+
4% NaCl	-	-	-	-	+	+	±	-	-	-	+	-	-	-	-	-
8% NaCl	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
a-D-glucose	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+
D-Mannose	+	+	+	-	+	+	±	+	-	-	-	-	+	+	+	+
D- Fructose	+	+	+	-	+	+	±	+	-	-	+	±	-	+	+	+
D-Galactose	+	+	+	-	+	+	+	+	+	-	±	-	+	+	+	+
3-Methyl Glucose	-	+	±	-	+	±	-	-	-	-	±	-	-	+	-	-
D-Fucose	-	+	+	-	+	-	±	-	-	-	±	-	-	±	-	-
L-Fucose	-	+	+	-	+	-	+	±	-	-	±	-	-	+	-	-
L-Rhamnose	+	+	-	-	+	+	+	+	-	-	±	-	±	+	-	+
Inosine	+	-	+	-	+	+	±	+	-	-	+	-	-	-	-	+
1% Sodium Lactate	-	-	+	+	+	+	+	+	-	+	+	±	-	+	+	-
Fusidic Acid	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
D-Serine	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	±
D-Sorbitol	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	+
D-Mannitol	-	-	+	-	+	+	+	-	-	-	±	-	-	+	+	+
D-Arabitol	-	±	+	-	+	±	+	-	-	-	±	-	-	±	-	-
Myo-Inositol	-	+	+	-	+	+	+	-	-	-	+	+	-	±	-	-
Glycerol	-	-	+	-	+	+	+	+	-	-	+	+	-	+	+	+
D-Glucose-6-PO4	+	+	+	-	+	-	±	±	+	-	+	-	+	+	+	-
D-Fructose-6-PO4	+	+	+	-	+	±	±	±	-	-	+	-	±	+	+	-
D-Aspartic Acid	-	-	+	-	+	+	+	-	-	-	-	±	-	-	-	-
D-Serine	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-
Troleandomycin	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-
Rifamycin SV	-	-	+	+	+	+	+	+	-	+	-	+	+	+	-	+
Minocycline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin	+	+	±	-	+	+	-	+	-	-	+	-	+	+	-	-
Glycyl-L-Proline	+	+	+	-	+	+	+	+	+	+	±	-	-	+	-	-
L-Alanine	-	+	+	-	+	+	±	+	-	-	-	+	-	+	-	-
L-Arginine	±	+	±	-	+	+	-	+	-	-	±	+	-	±	-	-
L-Aspartic Acid	±	+	+	-	+	+	+	+	-	-	+	+	+	+	-	-
L-Glutamic Acid	+	+	+	-	+	+	+	+	+	±	+	+	+	+	-	-
L-Histidine	+	+	+	-	+	-	-	+	-	-	±	+	-	±	-	-
L-Pyroglutamic Acid	+	+	+	-	+	±	+	-	-	-	±	-	-	-	-	-
L-Serine	-	+	+	-	+	+	-	±	-	-	+	+	-	-	-	-
Linocomycin	-	+	+	-	-	+	+	+	+	+	-	+	+	+	-	+
Guanidine HCl	-	-	-	-	+	+	+	-	-	-	+	-	-	-	-	+
Niaproof 4	-	-	-	-	-	-	±	-	-	-	-	-	-	-	-	-
Pectin	-	+	-	-	+	+	-	+	-	-	-	-	-	+	+	+
D-Galacturonic Acid	+	+	-	-	+	-	+	+	-	-	-	-	+	+	-	-
L-Galactonic Acid Lactone	+	+	±	-	+	-	±	-	-	-	-	-	+	+	-	-
D-Gluconic Acid	+	+	+	-	+	+	+	+	+	-	+	-	-	+	-	+
D-Glucuronic Acid	+	+	+	-	+	+	+	+	-	-	-	-	-	+	-	-
Glucuronamide	+	+	+	+	+	-	±	±	-	±	±	±	-	+	-	-
Muric Acid	+	+	+	-	+	±	+	+	-	-	±	-	+	-	-	-
Quinic Acid	-	-	+	-	+	+	+	-	-	-	±	+	-	+	-	-
D-Saccharic Acid	+	+	+	-	+	+	+	+	-	-	±	-	+	+	-	-
Vancomycin	-	-	+	+	-	+	+	-	-	+	-	+	-	-	-	±
Tetrazolium Violet	-	-	+	-	-	-	+	-	-	-	-	-	-	+	-	±
Tetrazolium Blue	-	+	+	-	+	-	+	±	-	+	-	+	-	+	-	-
p-Hydroxy-Phenylacetic Acid	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-
Methyl Pyruvate	+	+	+	+	±	±	+	+	-	+	+	+	+	±	-	+
D-Lactic Acid Methyl Ester	-	±	-	-	+	-	-	-	-	-	±	-	-	-	-	-
L-Lactic Acid	+	+	+	+	+	±	+	-	+	+	+	+	-	+	-	+
Citric Acid	-	-	+	-	+	+	+	+	+	-	-	-	+	+	-	-
a-Keto-Glutaric Acid	-	+	+	-	+	-	+	±	+	+	+	-	+	+	-	+
D-Malic Acid	-	+	-	-	+	-	+	-	-	-	±	-	+	+	-	-
L-Malic Acid	+	±	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Bromo-Succinic Acid	+	+	±	±	+	+	+	+	-	-	+	-	-	+	-	-
Nalidixic Acid	-	-	+	±	-	+	+	-	-	-	-	-	+	-	-	+
Lithium Chloride	-	-	-	-	+	+	-	-	-	-	+	-	-	-	+	+
Potassium Tellurite	-	-	±	-	+	+	+	-	-	-	+	+	-	-	+	+
Tween 40	+	+	+	-	+	±	-	±	±	-	+	-	+	+	-	-
y-Amino-Butyric Acid	-	-	+	-	+	+	+	-	-	-	-	+	-	-	-	-
a-Hydroxy-Butyric Acid	-	-	±	-	±	-	±	-	-	-	-	±	-	±	-	+
b-Hydroxy-D, L-Butyric Acid	+	+	+	+	±	-	+	+	-	-	±	+	+	+	-	-
a-Keto-Butyric Acid	+	+	+	-	±	-	+	+	-	-	±	±	-	+	-	+
Acetoacetic Acid	-	-	-	±	+	+	±	±	±	-	+	+	±	+	-	+
Propionic Acid	-	-	+	+	+	-	±	+	-	-	±	+	±	+	-	-
Acetic Acid	-	-	+	+	+	-	+	+	+	+	+	+	+	+	-	-
Formic Acid	-	-	+	+	±	-	+	-	±	-	+	-	-	-	-	-
Aztreonam	-	+	-	-	+	+	+	±	+	+	+	-	+	+	-	+
Sodium Butyrate	-	-	-	-	+	+	±	±	-	±	+	-	-	-	+	+
Sodium Bromate	-	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-

Author statement

All authors approved the submission of this manuscript to the International Journal of Systematic and Evolutionary Microbiology. The authors contributed as follows: RR and VP conducted the experiments, RR, VP and HB conceptualized the project, analyzed the experiments, and validated the results, and wrote, reviewed and edited the manuscript,

HB supervised the experiments, and was responsible for the acquisition of the financial support.

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Ethical statement

No experimental work that included human or animal subjects was conducted.

Conflicts of interest

The authors declare that there are no conflicts of interest.

CHAPTER 6

Harnessing Soil Microbes to Improve Plant Phosphate Efficiency in Cropping Systems

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Abstract

Phosphorus is an essential macronutrient required for plant growth and development. It is central to many biological processes, including nucleic acid synthesis, respiration, and enzymatic activity. However, the strong adsorption of phosphorus by minerals in the soil decreases its availability to plants, thus reducing the productivity of agricultural and forestry ecosystems. This has resulted in a complete dependence on non-renewable chemical fertilizers that are environmentally damaging. Alternative strategies must be identified and implemented to help crops acquire phosphorus more sustainably. In this review, we highlight recent advances in our understanding and utilization of soil microbes to both solubilize inorganic phosphate from insoluble forms and allocate it directly to crop plants. Specifically, we focus on arbuscular mycorrhizal fungi, ectomycorrhizal fungi, and phosphate-solubilizing bacteria. Each of these play a major

role in natural and agroecosystems, and their use as bioinoculants is an increasing trend in agricultural practices.

Introduction

Phosphorus (P) is a major element that is present in all soils worldwide in both inorganic and organic forms. The inorganic forms are derived from the weathering of primary mineral rock, including various forms of apatite (Rodriguez & Fraga, 1999). Upon weathering, inorganic P (P_i) exists in the soil solution as orthophosphates, including $H_2PO_4^-$ and HPO_4^{2-}. Depending on soil conditions, orthophosphates can be adsorbed to mineral complexes, converting them into insoluble forms (Kruse et al., 2015). In acidic soils, they are bound to both iron and aluminum oxides as well as clay minerals; however, in alkaline soils, they are bound mainly to calcium carbonate (Hinsinger, 2001; Matar, Torrent, & Ryan, 1992). In addition, soluble orthophosphates that are not adsorbed are often assimilated into various biological systems; hence, approximately 30–80% of the P present in soil is immobilized in various organic forms (Dalai, 1977). The most common form of organic P is inositol phosphate, which makes up approximately 60% of the total organic P in the soil (Dalai, 1977). Inositol phosphate is stable and is formed by a series of phosphate esters ranging from monophosphates to hexaphosphates. Phytate, or inositol hexakisphosphate, is the most abundant form of inositol phosphate (Turner, Paphazy, Haygarth, & McKelvie, 2002). Additional forms of organic P include glycerol phosphates, phospholipids, nucleic acids, and sugar phosphates; all of these are mainly contained in soil microbial biomass and combined make up less than 2% of the total soil organic P (J. W. Stewart & Tiessen, 1987). All forms of organic P in the soil are not bioavailable, i.e. not

readily available for plant uptake, because they have inherently high molecular weights and therefore must be hydrolyzed into soluble inorganic forms, such as orthophosphate (J. W. Stewart & Tiessen, 1987). To summarize, although the soil contains large quantities of P, most of it is not available for plants, forcing them to develop different strategies to acquire P, including the establishment of symbiotic associations with soil microbes (Figure 17).

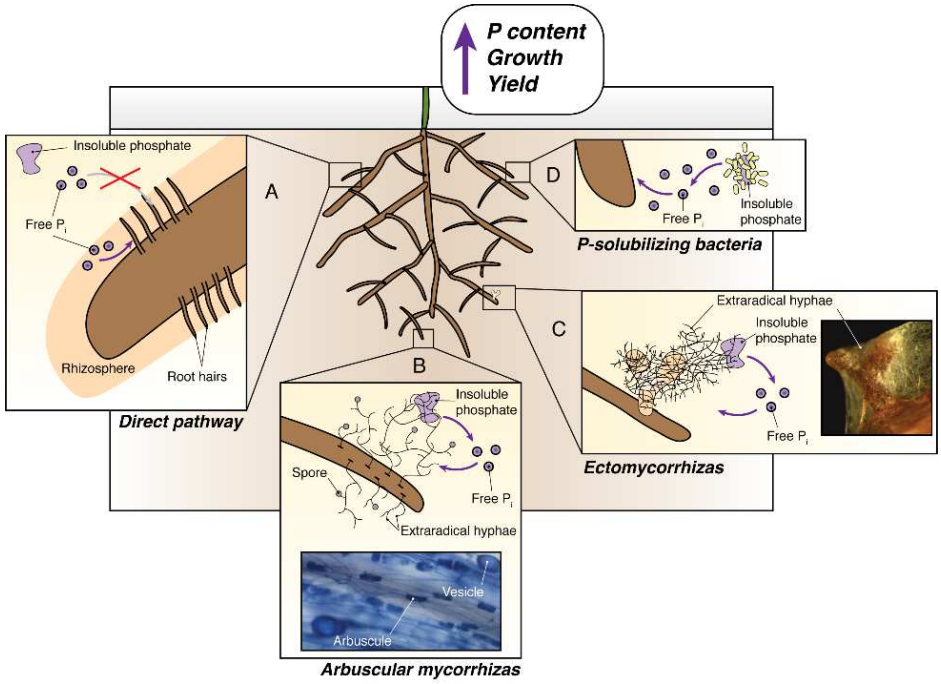

Figure 17. Schematic model showing the acquisition of phosphorus in plants, facilitated by arbuscular mycorrhizal fungi, ectomycorrhizal fungi, and P-solubilizing bacteria. (A) Root hairs on the roots of plants are able to acquire inorganic phosphate (Pi) from soil that is within close proximity (e.g., within the rhizosphere). This method of Pi acquisition is known as the direct uptake pathway. Insoluble forms of phosphate and free Pi that are outside of the rhizosphere cannot be reached by the plant (red cross). (B) Spores of arbuscular mycorrhizal fungi germinate, and emerging hyphae make contact with and penetrate roots in order to form arbuscules and sometimes vesicles in plant cortical cells. Extraradical hyphae exploring the soil can extract Pi from insoluble forms of phosphate (e.g., minerals and organic matter) and acquire it. Pi is then transported through the hyphae colonizing the roots and delivered to plant cells. (C) Specific plant–fungal structures called ectomycorrhizas can be formed on woody plant roots. As with arbuscular mycorrhizas, extraradical hyphae exploring the soil extract Pi from insoluble forms and acquire it. These hyphae can sometimes form rhizomorphs that are highly differentiated structures (not represented here). Pi is transported towards the roots and then delivered to plant cells. (D) Insoluble forms of phosphate are actively degraded by P-solubilizing bacteria. The resulting free Pi can either be taken up by the roots or other organisms, including mycorrhizal fungi. The improvement of P acquisition through direct or indirect microbial pathways leads to the increase of P content in aboveground tissues (e.g., seeds and fruits), thus increasing plant growth and yield.

In plants, P is a fundamental component of nucleic acids, phospholipids, and energy-shuttling nucleotide triphosphates. As such, P plays a crucial role in many molecular

processes, including nucleic acid synthesis, membrane synthesis and stability, respiration, signal transduction pathways, enzymatic activity, and redox reactions (Cade-Menun, Carter, James, & Liu, 2010; Ha & Tran, 2014). P is also vital for supporting photosynthesis, embryo and seed formation, and biological nitrogen fixation (Ha & Tran, 2014; Kouas, Labidi, Debez, & Abdelly, 2005). Due to the pivotal role of P in plants, its optimal concentration often exceeds 60 μmol g^{-1} of dry mass (Vance, Uhde-Stone, & Allan, 2003). However, as described above, the concentration of P in the soil that is bioavailable to plant roots is quite low, ranging from 1 to 10 μM in the form of P_i (Bieleski, 1973). Thus, when the soil P supply falls short of the plant P demand, it results in multiple adverse effects on plant growth and development, including defoliation, intolerance to biotic and abiotic stressors, and significant yield reduction (Jain et al., 2007; Sanchez-Calderon et al., 2005).

To combat yield reduction and meet the demand for food of the growing human population, chemical fertilizers, including monoammonium phosphonate, diammonium phosphate, superphosphate, and different formulations of NPK, have been used extensively worldwide since the 1960s to provide additional P (Carvalho, 2006). Although this practice has proven effective in temporarily treating the symptoms of P nutrient deficiency in crops, frequent applications of excess P fertilizers have detrimental environmental impacts, including the eutrophication of aquatic ecosystems due to the leaching and runoff of residual P from agricultural fields (Cade-Menun et al., 2010; P. Gyaneshwar et al., 2002). In addition, P is a non-renewable resource, and the known rock phosphate reserves that currently feed the agronomic P fertilizer demand are

unsustainable and estimated to be depleted within 50–100 years (Cordell et al., 2009). Furthermore, rock phosphate also contains heavy metal residues such as arsenic, chromium, lead, mercury, nickel, vanadium, and cadmium, thus contributing to the accumulation of heavy metals in arable land (Rutherford et al., 1995). Finally, between 75 and 90% of the applied P is precipitated by iron, aluminum, or calcium complexes present in the soil, making it unavailable to the plant in the first year of application (Cade-Menun et al., 2010; Gyaneshwar et al., 2002). Poor soil fertility and high P applications also directly inhibit microorganisms that can naturally improve P availability in soils (Garcia, Delaux, Cope, & Ane, 2015).

Plants adapted to P-deficient soils improve their P acquisition and use efficiency by 1) changing root architectural traits, 2) increasing the rate of P mineralization of rock minerals, 3) remobilizing organic sources of P via hydrolysis, 4) replacing phospholipids in membranes with sulfo- and galactolipids, or 5) employing metabolic pathways that can conserve P (Duff, Sarath, & Plaxton, 1994; Hinsinger, 2001; Jones, 1998). The most important adaptation to P limiting soil conditions, however, is the development of interactions with soil microbes, particularly mycorrhizal fungi (Raven, Lambers, Smith, & Westoby, 2018). In this review, we describe how plant-associated soil microbes mobilize and deliver P to their host plants. In particular, we summarize key information about how arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) fungi, as well as phosphate solubilizing bacteria contribute to plant P nutrition. We also describe how these microbes could serve as a sustainable alternative to synthetic P fertilizers used in agricultural and forestry production.

The Use of Arbuscular Mycorrhizal Symbiosis in Agriculture to Improve Phosphate Uptake

Plants are able to acquire P_i from the soil solution either directly via their own root system, particularly through root hairs (Figure 17A), or indirectly via their symbiotic association with mycorrhizal fungi (Figures 17B,C). Due to the slow rate of P_i diffusion in soils, the direct uptake pathway only allows plants to access P_i from the rhizosphere (Kraus, Fusseder, & Beck, 1987; S. E. Smith, Anderson, & Smith, 2015). Root hairs play a crucial role in expanding the root surface area involved in nutrient uptake, especially under low P conditions (Bates & Lynch, 1996). High-affinity transport proteins localized to the outer surface of root epidermal cells absorb P_i from the soil solution against the concentration gradient. The activity of these transporters is dependent on the active acidification of the soil solution by the plant, which allows the simultaneous co-transport of P_i with hydrogen ions into the plant cell (Rausch & Bucher, 2002).

In natural ecosystems, most land plants also acquire P through the indirect or mycorrhizal uptake pathway via the extension of their root system through associations with various classes of mycorrhizal fungi. One class includes AM fungi, which are ubiquitous in soils around the globe. They belong to the phylum Mucoromycota, sub-phylum Glomeromycotina, and form a symbiotic association with more than 70% of land plant species, including many agronomically important crops (Sawers et al., 2017; S. E. Smith & Smith, 2011). Among these major crop cereals, legumes, grasses, and trees can be found colonized by AM fungi in the field (B. Wang & Qiu, 2006). Many key plant genes required to form AM associations, and absent from non-mycorrhizal plants, have

been identified in model angiosperms in the past few decades (Vigneron, Radhakrishnan, & Delaux, 2018). Although AM fungi are ubiquitous, obligate and facultative mycorrhizal host plants vary in their degree of specificity when recruiting AM fungal partners (Sepp et al., 2019). AM fungi explore the soil for water and mineral nutrients through the development of an extensive extraradical mycelium. The collective soil volume surrounding all extraradical hyphae is referred to as the "mycorrhizosphere"(Timonen & Marschner, 2006). Compared to the rhizosphere, the volume of the mycorrhizosphere is orders of magnitude larger, given that the extraradical hyphal networks of AM fungi can extend up to 25 cm from the plant root into the soil (S. E. Smith et al., 2015). Inside the root, AM fungi form a network of intercellular hyphae and intracellular nutrient exchange structures in the root cortex, called arbuscules (Harrison, 2005) and, in some cases, storage structures called vesicles. Plants transfer photosynthetic carbon in the form of sugars (Baier et al., 2010) and lipids (Luginbuehl et al., 2017) across the periarbuscular and mycorrhizal interface to the fungal partner (Kiers et al., 2011; C. Y. Zheng, Ji, Zhang, Zhang, & Bever, 2015). In return, AM fungi provide the plants with water and various nutrients, such as P, nitrogen, sulfur, and trace elements (Garcia, Doidy, Zimmermann, Wipf, & Courty, 2016). It is well documented that AM fungi contribute significantly to the P_i nutrition of their host plant, particularly under P_i limitation (S.E Smith & Read, 2008). Soil exploring hyphae excrete enzymes that mineralize or mobilize plant unavailable organic and inorganic forms of phosphate (Figure 17B). As soon as P_i is taken up into the fungal cytoplasm, it can replenish the metabolically active P pool within the hyphae, or it is further polymerized

into polyphosphates that are stored in vacuoles, transported towards the intraradical hyphae, and delivered to the plant host through specific fungal and plant transport proteins at the arbuscular interface (Guerrero-Galan et al., 2018). Most of the P_i transporters involved in the direct pathway are downregulated when roots are colonized by AM fungi, and instead mycorrhiza inducible Pi transporters are expressed in the periarbuscular membrane, indicating a shift from the direct P uptake pathway towards the mycorrhizal one (Paszkowski, Kroken, Roux, & Briggs, 2002).

Among all crops, only legumes have the ability to interact with both AM fungi and the symbiotic nitrogen-fixing bacteria rhizobia. This dual association results in the formation of a unique and highly beneficial tripartite interaction (Afkhami & Stinchcombe, 2016). Rhizobia are housed by plants in specialized root nodules that provide an environment conducive for bacteria to convert atmospheric nitrogen (N_2) into a plant usable form (i.e., ammonium), which is then provided to the plant (Desbrosses & Stougaard, 2011). In return, host plants allocate photosynthetic carbon to rhizobia residing in the nodules (Paul & Kucey, 1981). Biological nitrogen fixation can satisfy more than 70% of a host plants' nitrogen demand (Herridge, Peoples, & Boddey, 2008) and contributes to one third of our global agricultural nitrogen needs (J. G. Liu, Ma, Ciais, & Polasky, 2016). Converting one molecule of N_2 into plant assimilable ammonium is an energy-expensive reaction requiring 16 molecules of ATP (Udvardi & Poole, 2013). Therefore, roots with functional nodules need substantially more P_i than non-nodulated roots (Sulieman, Schulze, & Tran, 2013). Legumes growing in P_i-limited environments are only poorly nodulated or have reduced nodule growth (Schulze,

2004). In sub-Saharan Africa field experiments, P_i supplements increased soybean seed yields after an inoculation with rhizobia, highlighting the need for proper P_i nutrition in legume cultivation (Thuita, Vanlauwe, Mutegi, & Masso, 2018). Due to their positive impact on P nutrition, AM fungi facilitate biological nitrogen fixation in P-deficient soils (Bournaud et al., 2018; Puschel et al., 2017). Thus, legumes forming tripartite interactions have accelerated growth, increased photosynthetic and biological N_2 fixation rates, and higher nutrient contents than plants colonized by only one root symbiont (Ibiang, Mitsumoto, & Sakamoto, 2017; Mortimer et al., 2013; X. R. Wang, Pan, Chen, Yan, & Liao, 2011).

The majority of agricultural crops develop symbiotic associations with AM fungi; as such, it is crucial for breeding programs to retain this trait within the germplasm of crop species. Retaining the ability of crop species to associate with AM fungi is particularly important, because recent studies revealed that the application of AM fungi to field conditions can improve the growth response and seed yield of some crops, including alfalfa and soybean (Eulenstein et al., 2017; Ortas, 2012). For example, soybean plants that were inoculated with AM fungi, showed similar P contents and seed yields, and had a higher fertilization efficiency than fertilized control plants (Cely et al., 2016). The identification of highly compatible AM fungal strains, in combination with management choices, will make the fungal communities durable, thus conferring continuous benefits to host plants; in contrast, when chemical fertilizers are applied to a field, they provide short-term benefits necessitating annual application (Verbruggen, van der Heijden, Rillig, & Kiers, 2013). Although AM fungi can be seen as an exciting alternative to minimize crop

dependency on P fertilizers, their actual impact is currently strongly debated by several groups since contrasting effects of AM colonization on plant P nutrition have been reported (Ryan & Graham, 2018). Indeed, many uncontrolled factors, including native AM fungal communities, can lead to a high variability in plant growth, nutritional or yield responses (Niwa et al., 2018). Nutritional and yield benefits of AM fungi also depend on the crop species or its variety, or the AM species or its fungal isolate (X., S., & Bucking, 2016). For instance, due to its large root system, wheat typically has a lower responsiveness to AM fungi than other crop species (H. Y. Li, Smith, Dickson, Holloway, & Smith, 2008). Altogether, this reflects the importance of intensifying our research efforts to identify crop and fungal combinations that lead to maximum benefits in agricultural systems.

The Use of Ectomycorrhizal Fungi to Improve Phosphate Uptake for Lignocellulosic Biofuel Crops

From an agronomic standpoint, woody plants are not traditionally grown as crops; however, extensive research has shown that some fast-growing species of woody plants (e.g., *Populus*) can be grown as feedstock for the production of lignocellulosic biofuels (Somerville, Youngs, Taylor, Davis, & Long, 2010). For this reason, here we consider the production of lignocellulosic biofuels from an agronomic perspective. Breeding programs have already developed cultivars within the genus *Populus* that are optimized for biomass yield (M. Guo et al., 2015). In addition, genetically modified *Populus* lines have been developed with decreased cell wall recalcitrance, thus allowing for greater biomass conversion to bioethanol (Macaya-Sanz et al., 2017). Although breeding and molecular

approaches have increased biomass yield and improved its conversion to biofuel, respectively, the agroecological context in which perennial biofuel crops will be grown has not been fully evaluated. The term “marginal lands” is used to describe low quality land that is not suitable for the production of food or feed from an economic standpoint, but that could serve as land where adapted biofuel crops can be produced (Richards, Stoof, Cary, & Woodbury, 2014). Although the unsuitability can be due to many edaphic factors, one crucial factor is nutrient availability (Alteri, 2002). To produce sufficient biomass for biofuel production, adequate access to nutrients is essential, particularly since the short rotation coppices used for woody plant biofuel production can rapidly deplete mineral nutrient reserves in the soil (Mitchell, 1995). In temperate and boreal forest ecosystems, trees are often dependent on ECM fungi for obtaining mineral nutrients from the soil via the mycorrhizal uptake pathway (Figure 17C; (Becquer et al., 2019)). In this section, we describe how ECM fungi specifically contribute to P nutrition in forests and can therefore be used to promote the sustainable agronomic production of woody plant biomass for lignocellulosic biofuel production.

In contrast to AM fungi, ECM fungi belong to the fungal phyla Basidiomycota, Ascomycota, and Mucoromycota (sub-phylum Mucoromycotina) (Spatafora et al., 2016). ECM fungi only colonize 2% of plant species, the majority of which are woody plants. However, ECM woody plants are the primary flora of forest ecosystems that cover approximately 30% of the global terrestrial surface (Pan, Birdsey, Phillips, & Jackson, 2013). In temperate and boreal forest ecosystems, up to 95% of tree short roots are colonized by ECM fungi (Martin et al., 2001). These mycobionts can substantially improve

the ability of trees to acquire water and nutrients (Becquer et al., 2019) and actively participate in nutrient cycling and carbon sequestration (Clemmensen et al., 2013). In addition, ECM fungi contribute significantly to the abiotic and biotic stress resistance of their host. Due to the mycorrhizosphere, trees colonized by ECM fungi can extend their exploration in the soil up to multiple meters, depending on the species (S. E. Smith et al., 2015). In many cases, a large percentage of the nutrient absorbing and actively growing part of the root system is colonized with ECM fungi and enclosed by a fungal sheath that can represent an apoplastic barrier, thus limiting the tree's ability to acquire nutrients via the direct plant uptake pathway (Figure 17C). The hyphae developed by ECM fungi inside tree roots form a network called the Hartig net, where nutrients are exchanged between both partners. As with AM fungi, ECM fungi acquire P_i from the soil through specialized transporters (Garcia et al., 2013; Tatry et al., 2009), store it in the vacuoles in the form of polyphosphates (Torres-Aquino et al., 2017), transport it towards the Hartig net, and release it into the mycorrhizal interface through transport proteins (Becquer et al., 2018). ECM plants also express specific transporters to absorb P_i directly from the mycorrhizal interface (Loth-Pereda et al., 2011). The filamentous nature of the mycelia formed by ECM fungi, which sometimes aggregate into highly differentiated structures called rhizomorphs, makes them more efficient than the roots of trees at foraging for mineral nutrients, such as P (Rousseau, Sylvia, & Fox, 1994). Multiple studies with multiple tree and fungal species grown at varying P concentrations have demonstrated that plants colonized with ECM fungi acquire significantly more P_i than non-mycorrhizal plants (Aquino & Plassard, 2004; Jentschke, Brandes, Kuhn, Schroder, & Godbold, 2001).

Furthermore, while foraging in nutrient-rich patches within the soil substrate, tree species that associate with ECM fungi allocate more carbon towards the formation of extraradical hyphae than towards the production of roots (Chen et al., 2016).

ECM fungi are capable of mobilizing P from both inorganic and organic sources within the soil (Figure 17C). Previous studies under controlled laboratory and field conditions have shown that ECM fungi dissolve inorganic sources of P (e.g., the calcium phosphate-enriched clay apatite) via the excretion of organic acids (e.g., oxalic acid) that they produce using plant-derived carbon (Rineau et al., 2013). These studies indicate that the weathering of apatite by the fungus is driven by the allocation of photosynthates from the host plant to the fungus that facilitates the mobilization and uptake of P that can be delivered to the host plant.

In addition to inorganic sources of P, ECM fungi can also extract phosphate from various organic sources and can enzymatically digest inositol hexaphosphate through the production of phosphatases (Antibus, Sinsabaugh, & Linkins, 1992) (Figure 17C). Phosphatase activity increases as the concentration of P_i decreases in the soil substrate (Colpaert, VanLaere, VanTichelen, & VanAssche, 1997). In other studies, even more complex sources of organic P (e.g., leaf litter (Perez-Moreno & Read, 2000), pollen (Perez-Moreno & Read, 2001a), necromass from dead nematodes (Perez-Moreno & Read, 2001b), and even seeds (Tibbett & Sanders, 2002) were exploited by ECM fungi to liberate P_i and subsequently deliver it to the host plant. Interestingly, ECM fungi can also obtain P directly through cooperative associations with both living saprotrophic fungi—which decompose dead organic matter (Lindahl, Stenlid, Olsson, & Finlay, 1999)—and

mycorrhiza helper bacteria—which efficiently solubilize P and increase the colonization of the plant (Fontaine, Thiffault, Pare, Fortin, & Piche, 2016).

Due to the ability of ECM fungi to improve P availability for their host plant, ECM fungal inoculants have been used in the horticultural production of woody plants and for reforestation efforts (Trappe, 1977). The application of ECM fungal inoculants could be a sustainable approach to improve P availability when growing woody plants for lignocellulosic biofuel production, particularly on marginal lands with limited P. Future studies should focus on identifying ECM fungal species that provide multiple benefits to crop species used for lignocellulosic biofuel production on marginal lands with limited mineral nutrients such as P.

Phosphate Solubilizing Bacteria and Their Potential to Increase the Phosphate Acquisition of Crops

Besides mycorrhizal fungi, certain soil bacteria can also play a key role in the soil P cycle (Figure 17D). According to estimates, up to 50% of the bacteria in the soil are considered phosphate solubilizing bacteria (S. B. Sharma et al., 2013). The diversity of bacteria having phosphate solubilizing capabilities is quite high and includes one or more bacterial species from the following genera: *Bacillus* (Banik & Dey, 1982, 1983; Vazquez et al., 2000), *Pseudomonas* (Tani et al., 2011), *Arthrobacter* (Yi et al., 2008) *Enterobacter* (Hwangbo et al., 2003), *Kluyvera*, *Chryseomonas (Vazquez et al., 2000)*, *Vibrio*, *Xanthobacter*, *Micrococcus*, *Klebsiella*, and more (Ohtake et al., 1996). These bacterial species act on different sources of P and have diverse mechanisms for solubilizing P in soils.

The primary method that soil bacteria use to liberate P_i from complex sources is by producing organic acids (Halder & Chakrabartty, 1993; Katznelson & Bose, 1959). There are several ways by which organic acids can convert insoluble forms of P into bioavailable forms. These include creating acidic microsites that lower the pH thus releasing P from calcium ions, chelating metal ions that typically immobilize P, and occupying exchange sites on soil and mineral ions. Each of these methods increases the levels of P_i in the soil solution (Gyaneshwar, Kumar, & Parekh, 1998). Soil bacteria can also solubilize inorganic sources of P by releasing inorganic acids, protons, hydroxyl ions, siderophores, and CO_2 (Jiang et al., 2018).

Soil bacteria primarily mineralize organic P with extracellular enzymes. The main groups of enzymes include non-specific phosphohydrolases (also called phosphatases), phytases, phosphonatases, or C-P lyases. Phosphatases are divided into two groups, acidic or alkaline, and soil bacteria release the optimal phosphatase to solubilize organic P, depending on soil pH. Non-specific phosphatases are the most abundant type and can mineralize the majority of organic P in the soil (Ragot et al., 2017). Phytase functions in releasing P from phytic acid (inositol), and phosphonatases and C-P lyases facilitate the release of P bound in organophosphonates (Richardson & Hadobas, 1997). Higher levels of nitrogen or carbon lead to increases in the phosphatase activity, most likely due to an increase in microbial activity (Wei, Sun, Tian, Chen, & Chen, 2018); meanwhile, higher P availability leads to a decrease in phosphatase and phytase activity (Spohn, Treichel, Cormann, Schloter, & Fischer, 2015).

There is a growing interest in using P solubilizing bacteria as biofertilizers to increase the bioavailability of P in soils used for crop production and to enhance the efficiency of P fertilizer applications (Figure 17D). Increases in plant performance and/or yield after an inoculation with P-solubilizing bacteria have been shown for many agronomically important crop species, including corn (Zhao et al., 2014), soybean (Ku et al., 2018), wheat (Kumar, Bauddh, Barman, & Singh, 2014), rapeseed (Valetti et al., 2018), mung bean (Biswas et al., 2018), and tomatoes (Nassal et al., 2018). While the periodic application of P fertilizer has adverse effects on the structure and function of phosphate-solubilizing bacterial communities, the opposite is true when little or no superphosphate is applied (Samaddar et al., 2019).

In 2016, market researchers estimated that the global biofertilizer market was worth 787.8 million USD. Phosphate solubilizing bacteria accounted for approximately 15% of the global biofertilizer revenue, which was the second largest and fastest growing sector (Grand, 2018). These figures indicate that phosphate solubilizing bacteria have proven to be another viable option as biofertilizers and are increasingly being used commercially to increase the abundance of plant available P in the rhizosphere.

Conclusion

In this review, we described three major groups of microbes that participate in the cycling of P within the soil and that have the potential to enhance the plant P acquisition efficiency in agricultural and forestry ecosystems. However, the variability in responses after inoculation with different fungi or bacteria justifies the need to pursue additional research focused on identifying soil microbes that improve plant phosphate efficiency in

cropping systems. More importantly, AM fungi, ECM fungi, and phosphate solubilizing bacteria do not act independently, but affect each other's activities. For example, *Populus* trees associate simultaneously with AM and ECM fungi, and both fungal partners could benefit from P that is made available by phosphate solubilizing bacteria. In addition, the presence of these symbioses can also have an effect on the activity of other root symbioses, for example symbiotic nitrogen fixing rhizobia in the tripartite interactions of legumes (reviewed in (Kafle et al., 2018)). Interestingly, additive effects on plant performance due to multiple symbioses is currently under debate (Gibert, Tozer, & Westoby, 2019), showing the importance of pursuing efforts towards the investigation of these complex plant–microbe relationships. These types of studies increase our understanding of the microbial systems that exist in nature and will likely facilitate the development of microbial inocula that are more efficient and optimized for the nutritional needs of plants. In parallel, the knowledge gathered from studying multi-partite mutualistic associations must be considered in breeding programs in order to create crop cultivars that can interact more efficiently with soil microbes and thereby maximize the sustainable use of mineral resources in agricultural and forest ecosystems. Finally, with the current frantic race for the production of bioinoculants produced by an increasing number of start-up companies, there is a need for more stringent quality control checks that should be instituted along with comprehensive field trials to ensure the efficiency, reliability, and sustainability of these commercial products.

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CHAPTER 7

Identification of Phosphate Solubilizing Biofertilizers and the Key Organic Acid Utilized

Abstract

Phosphorous (P) fertilizer is a nonrenewable resource and the mining sources have been estimated to be depleted within a hundred years. The majority of P within the soil is in an insoluble form, and the P fertilizer applied to fields can become immobilized and tied up, making it unavailable to plants. Phosphate solubilizing bacteria (PSB) and plant growth promoting bacteria have been investigated as potential bioinoculants to help increase P solubility and plant growth, respectively. PSB have several mechanisms to solubilize soil P, however one of the most common methods is by producing organic acids. Bacterial isolates were cultured from three corn fields and screened for PSB capabilities as well as additional plant growth promoting abilities. Three cultures; *Enterobacter cloacae, Raoultella ornithinolytica,* and *Kosakonia* sp. were found to be strong phosphate solubilizers. *E. cloacae* and *Kosakonia* sp. increased plant growth and P uptake compared to the control. These two species produced the highest levels of succinic acid compared to the other isolates tested, suggesting that this organic acid played a key role in solubilizing the inorganic P. *E. cloacae* and *Kosakonia* sp. are promising bioinoculant candidates.

Introduction

Phosphorus (P) is the second most needed plant nutrient. It is needed in numerous life processes such as respiration, photosynthesis, energy transfer and

storage, signal transduction, cell division and elongation, nitrogen fixation, and much more (Kouas et al., 2005; Sashidhar & Podile, 2010; Zaidi et al., 2009). There is an adequate amount of P in the soil to sustain crop growth, however in some soil only 0.1% of the phosphorus is in a readably available form (Zou et al., 1992). Due to the low levels of soluble P, heavy rates of fertilizer are typically applied. Chemical fertilizers have played a significant role in increasing crop yields to secure food production increases; however, fertilizers have begun to reach their theoretical maximum potential for yield increases (Gyaneshwar et al., 2002). In addition, there are several negative impacts of mining, distributing, and applying of P fertilizer. First of all, phosphorus is a non-renewable resource and the current phosphorus reserves are estimated to be depleted within 100 years (Steen, 1998). Additionally, heavy metal contaminants are carried over from the mining process into the fertilizers that are applied to agricultural land (Rutherford et al., 1995). Furthermore, up to 75-90% of the P fertilizer becomes bound in the soil and is not able to be used by the plant (Gyaneshwar et al., 2002). Phosphorus in the soil can be found in organic and inorganic forms, but the plant typically only assimilates inorganic orthophosphate forms (Beever & Burns, 1980).

Phosphorus that is bound to inorganic material can be found in stratum rock such as apatite, hydroxyapatite and oxyapatite, and these forms are highly insoluble (Rodriguez & Fraga, 1999). Phosphorus is moderately insoluble when it complexes with cations in soil particles such as Fe, Al, and Ca (Richardson et al., 2009). The soil pH greatly affects what cation P binds to, for example in alkaline soils P complexes with Ca and in acidic soils P forms complexes with Al, Fe, or Mn (Goldstein, 1986). Phosphate

solubilizing bacteria (PSB) have the ability to solubilize by several different mechanism, however the most commonly accepted and researched method is through secreting low molecular mass organic acids (OA) (Rodriguez & Fraga, 1999). Specific organic acids that can solubilize P are gluconic, formic, malic, succinic, acetic, oxalic, citric, and lactic acid (Bakri, 2019; Topolska et al., 2013; Wei et al., 2018). OA have a couple different mechanisms to solubilize the P; either through chelation of the metal ions to release the phosphate, and/or lowering the pH and releasing the P-ion from the mineral by H^+ substitution (Goldstein, Braverman, & Osorio, 1999; Pradhan & Sukla, 2005). There are two main membrane bound enzymes in the glucose metabolism pathway; glucose dehydrogenase (*gcd*) and gluconate dehydrogenase (*gad*) (Miller et al., 2010). The Gcd protein requires a pyrroloquinoline quinone cofactor (*pqq*). Further genetic testing needs to be conducted to determine additional organic acid production genes, or a universal PSB molecular marker.

Approximately 50% of the P in the soil is in an organic form (Yadav & Verma, 2012). When the P is in an organophosphorus compound it needs to be mineralized by extracellular or transmembrane enzymes by PSB. Some of the exoenzymes used to mineralize organic P are phosphatase, phytase, and C-P lyase (Prabhu et al., 2018). These enzymes are able to hydrolyze organophosphorus compounds such as phytate, glycerol phosphates, phospholipids, nucleic acids, and sugar phosphates (Stewart & Tiessen, 1987; Turner et al., 2002). The Pho regulon is most commonly involved in the mineralization of the organic P. When the Pho regulon is activated due to low soluble P levels, it activates approximately 90 phosphate-starvation-inducible proteins (Wanner &

Chang, 1987). The two-component regulatory system of the Pho regulon are named PhoR-PhoB in *Escherichia coli* (Tommassen et al., 1982) or PhoR-PhoP in *Bacillus subtilis* (Hulett et al., 1994). Some of the key exoenzymes are; alkaline phosphatase (*phoA*) (Yagil, Bracha, & Silberstein, 1970), Phospholipase (*phoD*) (Yang & Roberts, 2002), phytase (*phyC*) (Zou et al., 2006), glycerophosphodiester (*glpQ*) (Larson & Vanloobhattacharya, 1988), 5'-nucleotidases (*ushA*) (Innes et al., 2001). The *phoU* gene plays a role in the regulation of internal levels of phosphate within the cell, by interacting with the PhoR-PhoP/B two-component regulatory system (Willsky & MH., 1980). The *pstSCAB* genes encodes a high-affinity, ABC-type, P transport systems (Yuan et al., 2006), and polyphosphate kinase (*ppk*) is responsible for the storage of inorganic P, by activating the reversible polymerization of the terminal phosphate of ATP into a long-chain polyphosphate (Masahiro et al., 1992).

Plant growth promoting bacteria (PGPB) have been shown to increase plant performance by several methods such as nitrogen fixation (Shabanamol et al., 2018), indole-3-acetic acid production (Nutaratat, Monprasit, & Srisuk, 2017), fungal suppression (Etesami & Alikhani, 2016), phosphate solubilization (Muthukumarasamy, Revathi, Vadivelu, & Aruri, 2017), and much more. Considering the inefficiencies and negative effects of additional fertilizer to cultivated land, we are proposing to harness the beneficial mechanisms of PGPB and utilize them as bioinoculants. The objectives of this study were to isolate and characterize phosphate solubilizing bacteria, as well as their additional plant growth promoting capabilities. Additionally, we examined the

mechanisms used by PSB to solubilize insoluble inorganic phosphorus and assessed if the PSB were able to promote soybean plant growth.

Methods

Sample Collections and Isolation

Maize plants were collected in October 2017 from three different locations. The roots were conserved with the soil remaining intact. The location of the three plants were 44.273262 -95.877869, 44.245564 -95.656073, and 44.243411 -95.996360. Cultures were isolated from the plant tissue, the bulk root soil, and the rhizosphere soil.

Endophyte isolates were extracted from the shoot, leaves, and maize kernels. Tissue samples were cut to ≤ 2 mm in size and placed into a metal enclosed strainer. Tissue samples were then emerged under 70% ethanol solution and dipped for 1 min, sterile water for 30 sec, 5% bleach for 1 min, and then four washes with sterile water for 30 sec each. Tissue samples were then removed from the metal strainer and cut into smaller pieces aseptically. A small amount of the cut and surface sterilized tissue was placed on the surface of Tryptic Soy Broth (TSB) semi-solid media. Isolates were incubated at 30 °C for 24 h, then streaked onto a Tryptic Soy Agar (TSA) plate and re-incubated as before. Colonies were continually streaked onto new plates and incubated until individual colonies were isolated.

Bacteria from bulk root soil were isolated by adding 1 g of soil to 9 ml of phosphate buffered saline (PBS), vortexing and then 10 µl of the solutions were spread

onto TSA. Plates were incubated at 30 °C for 48 h and colonies were re-streaked and incubated to isolate pure colonies.

The rhizosphere soil isolates were bacteria that were present directly on the root surface. Roots were cut from plants and soaked in water for 1 min and rinsed to remove any excess soil on the roots. The roots were then placed in tubes with 10 ml of PBS. The tubes were placed in the sonicator for 60 s, and then the roots were removed and placed into another tube with 10 ml of PBS and placed in the sonicator for another 60 s, and once more were placed in a clean tube of 10 ml PBS and placed in the sonicator for 1 min. The solution from the third tube was used for isolation, by spreading 100 µl onto TSA followed by incubation for 48 h at 30 °C. Isolates were continually re-streaked until individual colonies were formed.

16s Sequencing

Twenty bacteria were selected for 16S rRNA sequencing based on the phosphate plate assay results, and their variety in colony morphology. Isolates were prepared in a 1:1 mix of Lysogeny Broth (LB) and 65% glycerol and frozen at -80°C. GENEWIZ (South Plainfield, NJ) conducted the 16S rRNA sequencing using Applied Biosystems BigDye version 3.1. The reactions were then run on Applied Biosystem's 3730xl DNA Analyzer.

Phosphate Plate Screening

Pikovskayas agar (PVK) (HiMedia Mumbai, India) was used for the plate assay test. A total of 70 isolates were screened for their phosphate solubilizing abilities. Individual bacterial colonies were stabbed onto a PVK plate five times in a star

formation. Plates were incubated at 30 °C for five d. Colony diameter and clearing zones were measured to determine the phosphate solubilizing index using the following calculation: Phosphate Solubilizing Index = ((colony diameter + halo zone diameter) / (colony diameter)).

Phosphate solubilization quantification screening

The molybdate-blue phosphate quantification test (J. Murphy, 1962) was used to determine the amount of phosphate solubilization from each of the eight isolates. Isolates were grown in LB for 24 h in the 30 °C shaker at 150 rpm. After incubation 10 µl of each isolate was re-inoculated into three new 5 ml LB tubes and incubated as stated before. Each culture was washed three times by centrifuging 2 ml in the microcentrifuge at 6,000 rpm for 10 min, removing the supernatant and re-suspending in National Botanical Research Institute's Phosphate growth media, modified to remove any phosphorus sources (NBRIP(-P)). After the three washes, 2 ml of NBRIP(-P) was added to re-suspend the bacteria. Bacteria were normalized by diluting with phosphate buffer saline (PBS), modified with no phosphate, to an OD density of 0.10 at 600 nm. A 1L mixture of the NBRIP media (Nautiyal, 1999) was prepared and modified as follows; glucose (10.0 g), $MgCl_26H_20$ (5.0 g), $MgSO_47H_20$ (0.25 g), KCl (0.2 g), $(NH_4)_2SO_4$ (0.2g), $Ca_3(PO_4)_2$ (3.8 g), $FePO_4$ (0.1 g), and $AlPO_4$ (0.1 g). Then it was washed three times to remove any access soluble phosphate by centrifuging, pouring off the supernatant, and resuspending in NBRIP(-P). Then 100 µl of the normalized bacteria were added to 15 ml of NBRIP. Tubes were vortexed and incubated in the 30 °C shaker at 150 rpm for 48 h. After vortexing tubes, 1 ml was centrifuged at 10,000 rpm for 10 min. The supernatant

was used to measure the amount of soluble P colorimetrically in 96 well plates. Each well received 40 µl of the reagent and 10 µl of the isolate, instead of the typical 200 µl to ensure the values were within the linear curve, then the values were multiplied by 20. The molybdate blue reagent is composed of the following; 5N sulfuric acid (12.5 ml), 10 g ammonium molybdate in 250 ml of H_2O mix and add (3.75 ml), 0.132 g L-Ascorbic acid 0.1M in 7.5 ml H_2O and add (7.5 ml), 0.2743 g potassium antimonyl tartrate (PAT) in 100 ml of H_2O and add (1.25 ml). A standard curve was made by using KH_2PO_4 as the soluble P source with levels of 0, 0.5, 1.0, 2.0, 5.0, 8.0, 10.0 mg P/L. The 96 well plate was then measured at 882 nm and mg P/L was calculated based on the standard curve. The pH was measured from a composite of 3 technical replications for every biological replicate.

Motility screening

Motility was evaluated by utilizing the Motility Test Medium (Fisher Scientific Lenexa, KS) and 0.05 g/L of 2,3,5-Triphenyltetrazolium chloride (TCC) (Fisher Scientific Lenexa, KS). Sterile media was poured into tubes and cooled to harden. Tubes were inoculated with fresh, overnight, pure cultures. Sterile inoculation needles were used to stab the semi-solid media, in the center of the tube and halfway down the length of the tube. The isolates were incubated at room temperature for 5 d. If the bacteria were motile, there was pink/red growth throughout the media (++). If the color change only occurs in the area of the inoculation needle, then the bacterium is immobile (-). When the color pigment moved slowly and had minimal growth from the stab line, the organism was weakly motile (+).

Nitrogen Fixing Plate Assay

The nitrogen fixing plate assay was conducted utilizing Nitrogen Free Agar (NFa) (Kirchhof et al., 1997), modified by an addition of 15 g/L agar using a nitrogen free agar source. Each of the selected eight isolates were streaked onto the NFa and incubated at 30 °C for 24 h. The isolate was considered to be able to fix nitrogen if the bacteria grow on the nitrogen free plate and the bromothymol in the media changed from green to blue, indicating a pH change in the medium.

Indole-3-Acetic Acid Biosynthesis Assay

The biosynthesis of indole-3-acetic acid (IAA) was quantified for the isolates. Isolates were cultured overnight at 30 °C in the shaker incubator at 200 rpm. We mixed 500 µg/ml of L-tryptophan in sterilized LB broth and filtered the solution through a 1 L filter system (0.22 µm). Bacterial isolates were normalized to an OD of 0.10 at 600 nm, by diluting them with PBS. Then 100 µl were inoculated into 5 ml of the LB / L-Tryptophan solution and placed in the shaker incubator at 30 °C rotating at 200 RPM for 4 d. Three technical replicates of each isolate were made at this time. After 4 d, the tubes were then centrifuged at 10,000 rpm for 10 min at 4 ^{o}C, and the supernatant was transferred to new tubes. A 96-well plate was used to measure the absorbance, by pipetting 100 µl of supernatant into the wells and 200 µl of Salkowski's reagent (35% perchloric acid and 2% 0.5M $FeCl_3$). Then 1-1.5 drops of 85% phosphoric acid was added to each well and incubated at room temperature in open air for 30 min in the dark. Absorbance was measured at a wavelength of 530 nm, and standards were made by

dissolving IAA (MP Biomedicals Shanghai, China) in 1N NaOH and creating concentration of 0, 5, 10, 20, 50, 100, and 200 µg/ml. The IAA concentrations were then based on the standard curve.

Fungal Suppression

Fungal suppression was assessed by a plate assay for the isolates. The four fungal pathogens used in the plate assay were *Fusarium oxysporum, Fusarium proliferatum, Fusarium graminearum*, and *Bipolaris sorokiniana.* The fungi were grown on potato dextrose agar (PDA) plates and cores were taken from the outer edge of the mycelia. The plugs were placed in the center of a fresh PDA plate and each isolate was streaked with parallel lines 2 cm on either side of the fungal core. The plates were incubated at 30 °C for 24 h and then at room temperature for 24 h. Fungal suppression was scored visually measuring the fungal inhibition zones.

Phytase Plate Screening

A plate assay was used to determine if the selected eight isolates can mineralize phytate. The media was composed of 1.5% glucose, 0.1% phytic acid sodium salt hydrate (Sigma-Aldrich Darmstadt, Germany), 0.2% NH_4NO_3, 0.05% KCL, 0.05% $MgSO_4$, 0.03% $MnSO_4$, 0.03% $FeSO_4$, 2.0% agar. A straight line was streaked across the width of the plate for each isolate and incubated at 30 °C for 48 h. If the culture created a clearing zone around the bacterial growth, then the isolate was able to solubilize the phytic acid. If the clearing zone was greater than the width of the isolate growth it was designated (++), if the isolate created a clearing zone but it was narrower than the

bacterial growth diameter it was designated (+), and if there was no clearing zone it was negative for phytase production (-).

Acid Phosphatase Screening

To determine if the selected isolates could produce acid phosphatases in the presence of an organic compound phenolphthalein diphosphate. The method used was (Matos et al., 2017), but modified to use the National Botanical Research Institute's Phosphate growth media (NBRIP) (Nautiyal, 1999), with the phosphorus source removed (NBRIP(-P)). NBRIP(-P) was used instead of the TSB as stated in the method, due to the excess levels of P in TSB. To determine if the isolates could produce acid phosphatases to solubilize the phenolphthalein diphosphate, ammonium hydroxide was added, and the color change was observed. If the isolate turned pink as well as a pink clearing zone around the isolate it was considered positive for acid phosphatase (++), if the isolate turned pink but there was no pink clearing zone around the growth that isolate was considered borderline (+), and if the isolate did not turn pink at all it was negative for acid phosphatase production (-).

Full Genome Sequencing

Due to the high phosphate quantification values*, Enterobacter cloacae* and *Raoultella ornithinolytica* had their full genomes sequenced by isolating the DNA using the method in *Current Protocols in Molecular Biology* (K. Wilson, 2001) amended by adding 0.5 µL of 100 mg/mL RNase with 10% SDS and proteinase K and three DNA wash steps with 70% ethanol. Samples were centrifuged at 4°C. The DNA extraction quality

was determined by gel electrophoresis on 1% agarose. Nextera DNA flex library prep kit (Illumina, Inc., San Diego, CA, USA) was used to make the genomic DNA library. The Illumina's MiSeq platform producing 2 x 300 bp paired end reads were utilized for the DNA sequencing. Raw reads were downloaded from the Illumina BaseSpace and uploaded to Galaxy (Afgan et al., 2018). The FastQC v0.11.7 was used for quality control, Trimmomatic v0.36.5 to trim the adapters and to eliminate the reads below Q25 (paired end reads) (Bolger, Lohse, & Usadel, 2014), and Unicycler v0.4.1.1 for genome assembly (Wick, Judd, Gorrie, & Holt, 2017). The assembly quality was determined using QUAST v4.6.3 (Gurevich, Saveliev, Vyahhi, & Tesler, 2013), and the annotation was done using the Prokka v1.12.0 pipeline (Seemann, 2014) set to a minimum contig length of 200 bp. Galaxy (Afgan et al., 2018) was used for annotation and individual gene identification, and RAST (Overbeek et al., 2005) was used for annotation and identification of subsystem features.

HPLC

Samples from the phosphate solubilizing quantification screening assay were used to determine the organic acid levels. In the phosphate solubilizing assay each isolate had 3 biological replications and 3 technical replications. For the HPLC testing the three technical replications were combined equally, resulting in a total of 3 biological replications per isolate that were tested for organic acids. An UltiMate 3000 UHPLC chromatographic system (Thermo Scientific Dionex, USA) equipped with an autosampler and a Diode Array detector was used. The separation was carried out using a reversed-phase Zorbax Rx- C8 column (5 µm particle size, 4.6 × 150 mm) (Rockland Technologies

Inc., USA). The injection volume was 5 µl and the column oven temperature was programmed at 30 °C. The mobile phase was 0.01M H_3PO_4 with an isocratic program for 10 minutes at a flow rate of 1.0 ml/min. The chromatographic data were recorded and processed using Theromo Scientific Chromeleon 7.2 software. Quantification was achieved by the absorbance recorded at 205 nm.

The following standards were used; L -(-)-malic acid (≥99%), succinic acid (≥99%), citric acid (≥99.5%), formic acid (≥95%) and L -(+)-lactic acid (≥98%) (Sigma-Aldrich Darmstadt, Germany). For HPLC mobile phase, deionized water was used and o-Phosphoric acid, 85% (certified ACS) was purchased from Fisher Scientific.

Standard stock solutions of L -(-)-malic acid (1000 ppm) succinic acid (1000 ppm), citric acid (2000 ppm), formic acid (2000 ppm) and L - (+)-lactic acid (2000 ppm) were prepared in deionized water. Calibration standards were prepared using the standard addition method with the solution that was used as control. Malic, citric, formic and lactic acid calibration standards were prepared in concentrations of 5, 10, 25, 50, 100, 200 and 300 ppm in the solution that was used as control. Succinic acid calibration standards were prepared in concentrations of 50, 100, 200, 300, 400 and 500 ppm.

Calibration curves for formic, malic and lactic acid were constructed using the concentrations ranging from 10 ppm to 300 ppm (for malic acid the area of the control peak (as zero ppm) was also included in the calibration range), while for citric acid the concentrations range was from 5 ppm to 300 ppm. For succinic acid the concentration range was from 50 ppm to 500 ppm. The calibration curve for each compound was

made using the peak area (y) versus the concentrations. For formic acid, instead of using area height was used, due to peaks overlapping. For malic acid, the concentration in the sample was corrected using linear extrapolation. For other acids, no correction was required.

Soybean Greenhouse Phosphorus Test

The eight selected isolates were grown in TSB overnight and normalized to 0.05 at 600 nm. South Dakota Foundation Seed, lot# 416, soybean seeds were sterilized using bleach and chlorine gas for 12-14 hours. Then the normalized bacteria were added to the soybean seeds at a rate of 3 µL/seed and inverted several times to coat the seeds. The potting material was 70% washed sand and 30% perlite, with 0.078 gm $Ca_3(PO_4)_2$/L of growth media, mixed thoroughly. Potting material was pasteurized utilizing a steam cart. Three seeds were planted 3.5 cm deep per each 3 L pot, and after the majority of seeds had emerged pots were thinned to one plant per pot. Plants received water through an irrigation system every hour for twelve seconds. The trial had two controls, a full phosphate 0.5 mM P rate (highP) and a 10% phosphate fertilizer 0.05 mM P rate (lowP). The Hoagland fertilizer (Hoagland & Arnon, 1950) was used for both controls but amended for the low P rate control to only have 0.05 mM 1M KH_2PO_4, and an additional 0.45 mM of 1M KCL to compensate for the lower K rate. The plants received 10 ml of the appropriate Hoagland fertilizer rates twice throughout the trial, three weeks after planting and two weeks before harvest. Pots were randomized and there were 10 replications per treatment. After a month and a half, the plants were harvested. Plants

were dried in the oven for 4 d at 70 °C then dry shoot and root biomass was determined.

We repeated the soybean greenhouse trial exactly as previously stated, however there were 15 replications instead of 10. Root structure from this trial was conserved to acquire root architecture values. Roots were scanned for root architecture parameters using the WinRhizo software and Epson Dual Lens System Scanner.

Plant phosphorus levels

To determine the level of phosphorus in the plant the vanadomolybdophosphoric acid colorimetric method was use (Greenberg et al., 1992). The five largest plants from each treatment were tested. For shoot P levels, two top leaves and two bottom leaves were ground together, and for the root P levels a vertical section was removed from one half of the root. The samples were ground using a Precellys 24 tissue homogenizer (Bertin technologies Montigny-le-Bretonneux, France) and then a 30-60 mg sample was weighed out and 1 ml of 2N HCL was added and incubated for 2 h in a digital heatblock (VWR Radnor, PA) at 95 °C. Samples were vortexed and centrifuged at the highest setting for 10 min. The reaction is formed in a cuvette by combining 475 µl H_2O, 500 µl ammonium molybdate-vanadate reagent (RICCA Chemical Company Arlington, TX), and 25 µl of the sample supernatant. Phosphate standards (Ricca Chemical Company, Arlington, TX) were used to create a standard curve. The absorbance was determined at 436 nm, and the phosphorus levels were based on the standard curve and normalized to the amount of sample used.

Statistical analysis

To compare means, all data were statistically analyzed by two-way analysis of variance (ANOVA) using Minitab 16 Tukey's (honest significant difference) post-hoc non-parametric test. Normality of the data was assessed using the Anderson-Darling Normality Test in Minitab 16. was performed to test for nonnormality. Significant differences were calculated at the $P \leq 0.05$ significance level to compare means, results were considered to be statistically significant when $P \leq 0.05$. And to determine if there were any correlations between parameters, the Pearson correlation coefficient was determined using Minitab 16. Linear regression was used to determine the concentration of the organic acids, IAA biosynthesis, and P solubilization quantities.

Results

Isolating and screening PSB

Seventy isolates were cultured from the three corn plants. Of those 70 bacteria, 20 were from the rhizosphere, 22 from the bulk soil, and 28 were endophytic bacteria. Of the 28 endophytes, 5 were from isolated kernels, 12 from leaf, and 11 from shoot tissue. All 70 isolates were screened for P-solubilizing abilities by the PVK plate assay, and the phosphate solubilizing index was calculated. On PVK media, 3 of the rhizosphere samples were able to solubilize $Ca_3(PO_4)_2$ and create clearing zones, 10 from bulk soil isolates, and 6 endophytes. Table 22 shows the PVK phosphate solubilizing index from the 21 isolates that were selected for 16S rRNA sequencing.

Table 22. Identities of the soil and endophytic isolates based on 16SrRNA and running a BLAST on the sequence, and phosphate solubilizing index values based on the PVK plate assay.

ID	Closest related organism	Isolation	Query Coverage	Identity	Accession	PVK Index
M1So2-2	*Pantoea agglomerans*	Soil	88	99.47	NR 041978.1	3
M2So2-3	*Paenibacillus intestini*	Soil	88	98.93	NR 156979.1	2
M3So1*	***Pantoea agglomerans***	**Soil**	**88**	**99.25**	**NR 116751.1**	**3**
MSH4	*Pantoea ananatis*	Shoot	91	99.47	NR 026045.1	3
M2R1*	***Bacillus safensis***	**Rhizosphere**	**88**	**98.93**	**NR 113945.1**	**1**
Tc1R1	*Ochrobactrum pseudogrignonense*	Rhizosphere	90	99.36	NR 042589.1	1
Tc3R1	*Pantoea agglomerans*	Rhizosphere	94	98.83	NR 116751.1	3
Tc2So2-1*	***Bacillus velezensis***	**Soil**	**87**	**99.12**	**NR075005.2**	**0**
Tc3So1	*Pantoea agglomerans*	Soil	88	99.25	NR 116751.1	3
Tc3So2*	***Kosakonia radicincitans***	**Soil**	**92**	**98.1**	**NR 117704.1**	**0**
TcK4	*Curtobacterium flaccumfaciens*	Kernel	94	99.46	NR 025467.1	1
TcL2	*Kosakonia radicincitans*	Leaf	94	98.1	NR 117704.1	0
TcL4	*Pantoea ananatis*	Leaf	91	99.26	NR 026045.1	3
TcSH4	*Pantoea agglomerans*	Shoot	92	99.16	NR 041978.1	3
Tr1R2*	***Bacillus cereus***	**Rhizosphere**	**92**	**99.06**	**NR 115714.1**	**0**
Tr1So1	*Pantoea agglomerans*	Soil	88	98.94	NR 116751.1	3
Tr3R2	*Ochrobactrum pseudogrignonense*	Rhizosphere	95	99.57	NR 042589.1	1
Tr3R3*	***Enterobacter ludwigii***	**Rhizosphere**	**87**	**99.36**	**NR 042349.1**	**0**
Tr3So1	*Bacillus proteolyticus*	Soil	89	99.15	NR 157735.1	0
TrSH1	*Pantoea agglomerans*	Shoot	88	99.57	NR 116751.1	0
TrSH4*	***Klebsiella michiganensis***	**Shoot**	**83**	**99.19**	**NR 118335.1**	**2**

* Isolates selected for further testing

Identifying isolates

Twenty-one isolates were selected for 16S rRNA sequencing, we selected a variety of isolates with positive and negative clearing zones on the PVK media. Table 22 shows the closest related organism in the NCBI database based on the 16S rRNA sequence. The following genera were identified from the sequenced isolates; *Pantoea, Paenibacillus, Bacillus, Ochrobactrum, Kosakonia, Curtobacterium, Enterobacter, Klebsiella*. Of these bacteria, 7 isolates were selected for further PSB and PGPB testing, based on PVK clearing zones and genetic variability. The 7 selected isolates are identified in Table 22, and *Pseudomonas aeruginosa* ATCC 27853 was used in all tests as a positive control.

Motility screening

Using the Motility Media and the colorless dye TCC, we were able to determine if the bacteria were motile or not, based on the bacteria reducing the TCC to form formazan, an insoluble red pigment. There was a clear difference between the motile and non-motile bacteria as indicated in Table 23. Tc3So2, M3So1, Tr3R3and TrSH4 were motile, and M2R1, Tc2So2-1, and Tr1R2 were not motile. *P. aeruginosa* did not appear to be motile after 2 d but after 5 d weak motility was observed.

Table 23. Nitrogen growth on NFa media (+ or -), motility screening on Motility media (++) indicates strong motility, (+) indicates weak motility, and (-) indicates no motility, and IAA biosynthesis.

ID	Isolate	Nitrogen Fixing	Motile	µg IAA/ mL change from control
Tc3 So2	*Kosakonia sp.*	+	++	0.72 d
M3 So1	*Pantoea sp.*	+	++	14.81 c
M2 R1	*Raoultella ornithinolytica*	+	-	36.36 b
Tr3 R3	*Enterobacter cloacae*	+	++	10.52 c
Tc2 So2-1	*Bacillus sp.*	-	-	54.7 a
Tr1 R2	*Bacillus sp.*	-	-	15.31 c
TrSH4	*Klebsiella sp.*	+	++	0.67 d
ATCC 27853	*Pseudomonas aeruginosa*	+	+	0.18 d

Nitrogen fixation

Nitrogen fixation was assessed on nitrogen free media with bromothymol blue as a pH color indicator. All the isolates were able to grow on the media and increased the pH due to production of ammonia as indicated by the color change from green to blue, with the exception of the two *Bacillus* strains Tc2So2-1 and Tr1R2. The bacteria that grew on NFa and increased the pH are shown in Table 23.

Indole-3-Acetic Acid biosynthesis assay

IAA biosynthesis was quantified after 4 d of growth in LB containing L-tryptophan. After 4 d Tc2So2-1 produced a statistically (P≤0.05) higher amount of IAA than the other isolates. M2R1 then produced the second highest amount, which was statistically (P≤0.05) greater than the rest of the isolates. Based on the statistical results of the IAA quantification, Tc3So2, TrSH4, and *P. aeruginosa* are unable to produce IAA relative the negative control. The quantities of IAA biosynthesis after 4 d are shown in Table 23.

Fungal suppression

The fungal suppression plate assay was able to show the degree of inhibition each isolate had on the fungal growth of the four species. Table 24 indicates if the isolate was able to strongly suppress the growth (++), partially suppress the growth (+), and had no effect on the fungal growth (-). Only two bacteria had the ability to suppress the growth of all four fungal isolates, Tc2So2-1 and Tr1R2 the two *Bacillus* isolates. The *Kosakonia* (Tc3So2) and *Klebsiella* (TrSH4) isolates were not able to suppress any of the pathogens.

Table 24. Fungal suppression plate assay results, depicted by strong suppression (++), partial suppresion (+), and no suppression (-). Fungal isolates tested were *Fusarium oxysporum*, *Fusarium proliferatum, Fusarium graminearum*, and *Bipolaris sorokiniana*.

ID	Isolate	*F. graminearum*	*F. proliferatum*	*F. oxysporum*	*B. sorokiniana*
Tc3 So2	*Kosakonia sp.*	-	-	-	-
M3 So1	*Pantoea sp.*	+	-	-	-
M2 R1	*Raoultella ornithinolytica*	+	-	++	-
Tr3 R3	*Enterobacter cloacae*	-	+	-	+
Tc2 So2-1	*Bacillus sp.*	++	++	++	++
Tr1 R2	*Bacillus sp.*	++	++	++	++
TrSH4	*Klebsiella sp.*	-	-	-	-
ATCC 27853	*Pseudomonas aeruginosa*	-	-	-	+

Phytase plate screening

The ability to mineralize phytic acid was assessed by a plate assay with phytic acid as the only P source. Clearing zones around the bacterial growth indicate that the isolated can produce phytase to mineralize organophosphorus. Table 25 shows that three isolates were unable to mineralize phytic acid; Tr3R3, Tc2So2-1, and Tr1R2. *P. aeruginosa* was able to mineralize the greatest amount of phytic acid in the plate assay, while the other isolates only mineralized a moderate amount.

Table 25. Phytase and acid phosphatase production based on plate screening. Organic phosphate mineralization depicted by strong mineralization (++), week mineralization (+), and no mineralization (-).

Isolate	Isolate	Phytase Production	Acid Phosphatase	pH of triP solubilization assay
Tc3 So2	*Kosakonia sp.*	+	++	4.53 ef
M3 So1	*Pantoea sp.*	+	++	4.35 g
M2 R1	*Raoultella ornithinolytica*	+	++	4.49 f
Tr3 R3	*Enterobacter cloacae*	-	++	4.52 f
Tc2 So2-1	*Bacillus sp.*	-	-	5.18 c
Tr1 R2	*Bacillus sp.*	-	+	6.07 b
TrSH4	*Klebsiella sp.*	+	++	4.65 d
ATCC 27853	*Pseudomonas aeruginosa*	++	-	4.62 de
Control		-	-	6.18 a

Acid phosphatase screening

The ability for the isolates to mineralize phenolphthalein diphosphate was assessed in a plate assay. Acid phosphatase biosynthesis was determined based on the pH change causing a color change and a clearing zone. Only two isolates were unable to mineralize any phenolphthalein diphosphate; Tc2So2-1 and *P. aeruginosa* (Table 25). Tr1R2 was the only borderline acid phosphatase producing isolate, and the rest created evident clearing zones indicated by pink halos around the isolates.

Phosphate solubilization quantification screening

Quantification of phosphate solubilization was conducted with the molybdate-blue colorimetric method for the eight selected isolates. The insoluble phosphorus source was 0.38% $Ca_3(PO_4)_2$, 0.01% $FePO_4$, and 0.01% $AlPO_4$. Bacteria were grown in 50 ml tubes to allow for enough surface area for the bacteria to be in contact with the phosphorus. All media and bacteria were washed three times before inoculation to ensure there was no excess soluble phosphorus in the growth medium. The two *Bacillus* species (Tc2So2-1 and Tr1R2) were unable to solubilize any phosphorus, they were statistically ($P \leq 0.05$) the same as the negative control (Figure 18). Whereas *Kosakonia* (Tc3So2), *Enterobacter* (Tr3R3), and *Raoultella* (M2R1) were able to solubilize a statistically ($P \leq 0.05$) greater amount than all the other isolates including the positive control *P. aeruginosa.* The final pH of each isolate is shown in Table 25. The pH of all isolates was lower than the control, additionally the two isolates with the highest pH were Tr1R2 and Tc2So2-1.

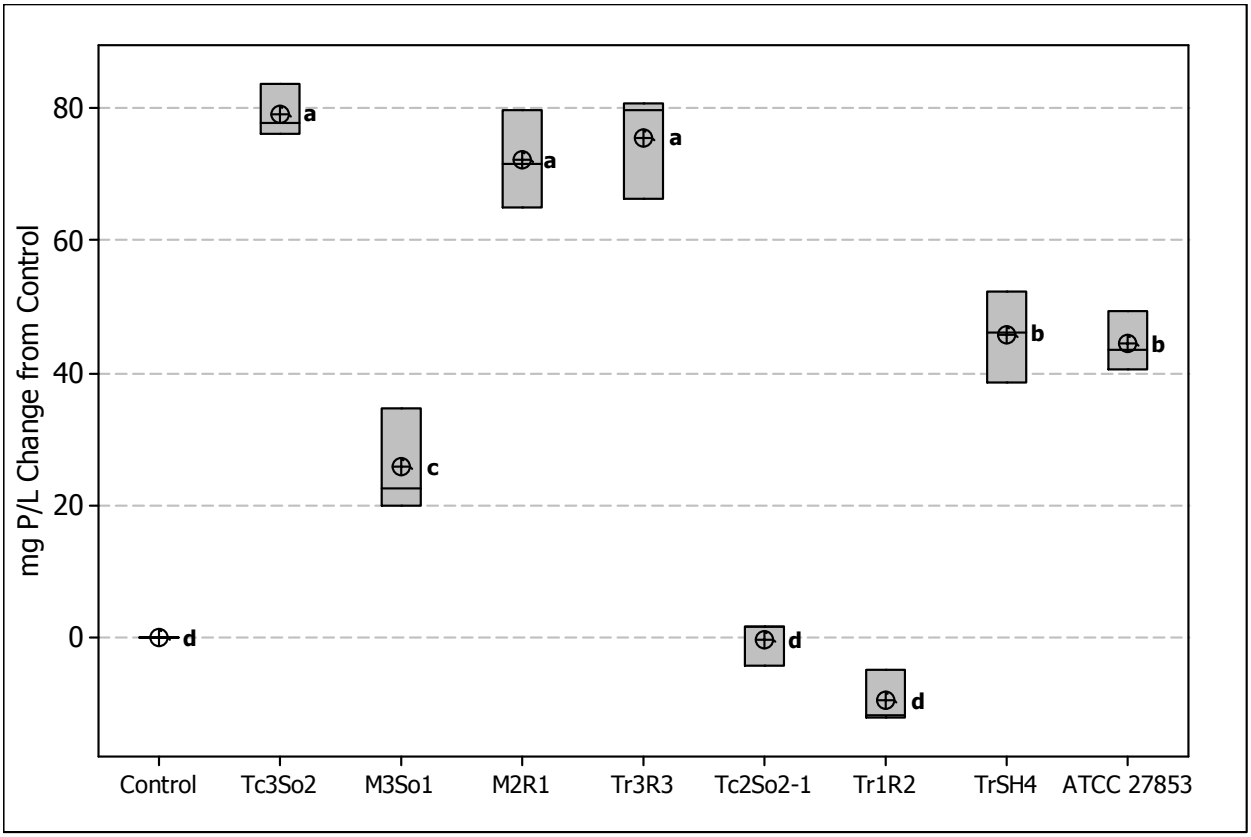

Figure 18. Boxplot of phosphate solubilizing quantification in mgP/L by the molybdate-blue method. Medians are indicated by horizontal lines and means indicated by crossed circle.

Full genome sequencing

Two or the three isolates (Tr3R3 and M2R1) that solubilized the greatest amount of phosphate had their full genomes sequenced. Through annotation using Galaxy (Afgan et al., 2018), we were able to identify if the two isolates of question possessed genes that could contribute to phosphate solubilization. We also used Rapid Annotation using Subsystem Technology (RAST) SEED Viewer (Overbeek et al., 2005) to annotate the genome and identify subsystem features within the genome contributing to the detected plant growth promoting abilities. Table 26 gives an overview of the identified

phosphate metabolizing genes in each genome depicted by Galaxy, and Table 27 identifies the plant growth promoting subsystem features assessed by RAST.

Table 26. Phosphate solubilizing genes annotated and identified using GALAXY for isolates Tr3R3 and M2R1. Tr3R3 represents species *Enterobacter cloacae* and M2R1 represents species *Raoultella ornithinolytica*.

Isolate	Description	Gene	Function	Product	Presence
Tr3R3, M2R1	Organic Acid production	Gcd	oxidize glucose to form gluconic acid	glucose dehydrogenase	Presence
Tr3R3, M2R1	Organic Acid production	Gad	oxidize glucose to form gluconic acid	gluconate dehydrogenase	Not Present
Tr3R3, M2R1	Organic Acid production	pqq A-F	Gad gene resides in the gene cluster pppA-F	pyrroloquinoline quinone	Not Present
Tr3R3, M2R1	Exoenzyme production	PhoR-PhoP	two main regulatory proteins of the Pho regulon	PhoR=Phosphate regulon sensor protein PhoR, PhoP= Transcriptional regulatory protein PhoP	Presence
Tr3R3, M2R1	Exoenzyme production	PhoR-PhoB	two main regulatory proteins of the Pho regulon	PhoR=Phosphate regulon sensor protein PhoR, PhoB= Phosphate regulon transcriptional regulatory	Presence
Tr3R3, M2R1	Exoenzyme production	PstS	Pi-specific transporters, most conserved of Pho	Phosphate-binding protein PstS	Presence
Tr3R3, M2R1	Exoenzyme production	PstC	Pi-specific transporters, most conserved of Pho	Phosphate transport system permease protein	Presence
Tr3R3, M2R1	Exoenzyme production	PstA	Pi-specific transporters, most conserved of Pho	Phosphate transport system permease protein	Presence
Tr3R3, M2R1	Exoenzyme production	PstB	Pi-specific transporters, most conserved of Pho	Phosphate import ATP-binding protein PstB	Presence
Tr3R3, M2R1	Exoenzyme production	PhoU	homeostasis of cellular phosphate modulation by the PstSCAB transporter	Phosphate-specific transport system accessory	Presence
Tr3R3, M2R1	Exoenzyme production	ppk	storage of inorganic P	Polyphosphate kinase	Presence
Tr3R3, M2R1	Exoenzyme production	PhoA	phosphatase released by Pho	Alkaline phosphatase	Presence
Tr3R3, M2R1	Exoenzyme production	PhoD	Phospholipases released by Pho	Phospholipases	Not Present
Tr3R3, M2R1	Exoenzyme production	PhyC	Phytase released by Pho	Phytase	Not Present
Tr3R3, M2R1	Exoenzyme production	GlpQ	Glycerophosphodiester released by Pho	Glycerophosphodiester phosphodiesterase, periplasmic	Presence
Tr3R3, M2R1	Exoenzyme production	UshA	5' nucleotidases released by Pho	Protein UshA	Presence
Tr3R3, M2R1	Exoenzyme production	aphA	acid phosphatase	Class B acid phosphatase	Presence

Table 27. Counts of plant growth promoting subsystem features annotated and identified using RAST SEED Viewer for isolates Tr3R3 and M2R1. Tr3R3 represents species *Enterobacter cloacae* and M2R1 represents species *Raoultella ornithinolytica.*

Isolate	Subsystem Feature	Count
Tr3R3	Motility	15
M2R1	Motility	0
Tr3R3	Invasion and intracellular resistance	17
M2R1	Invasion and intracellular resistance	17
Tr3R3	Siderophores	27
M2R1	Siderophores	29
Tr3R3	Quorum sensing and biofilm formation	14
M2R1	Quorum sensing and biofilm formation	9
Tr3R3	Auxin biosynthesis	5
M2R1	Auxin biosynthesis	5
Tr3R3	Nitrogen Metabolism	36
M2R1	Nitrogen Metabolism	36

HPLC

Organic acid levels are shown in Table 28, based on the HPLC results. Succinic was the most frequently produced organic acid. Formic acid was produced at a relatively low levels for all isolates. Malic acid was only produced by Tr3R3 and *P. aeruginosa*, however *P. aeruginosa* produced more OA, potentially due to the width of the peak. Lactic and citric both had wide ranges of acid concentrations, with M3So1 and Tc3So2 showing the highest lactic acid values, and Tr3R3 the highest citric acid value. Of the isolates that produced succinic acid, Tr3T3 and Tc3So2, produced significantly ($P \leq 0.05$) higher levels than the other isolates. Statistical differences based on linear regression estimates from the calibration curve.

Table 28. Concentration of organic acids in ppm based on HPLC analysis.

Concentration of acids in ppm													
ID	Isolate	Formic		Malic		Lactic		Citric		Succinic		Estimated Totals
Tr1R2	*Bacillus sp.*	ND		ND		Below detection limit (6.26)	b	ND		ND		6.26
Tr3R3	*Enterobacter cloacae*	Below detection limit (3.22)	a	54.18	b	ND		104.69	a	384.49	a	546.58
TrSH4	*Klebsiella sp.*	Below detection limit (5.29)	a	ND		ND		9.56	b	192.25	b	207.10
M2R1	*Raoultella ornithinolytica*	ND		ND		ND		72.85	d	122.24	c	195.09
M3So1	*Pantoea sp.*	Below detection limit (9.44)	a	ND		210.75	a	ND		192.81	d	413.00
Tc2So2-1	*Bacillus sp.*	ND		ND		Below detection limit (7.13)	b	ND		ND		7.13
Tc3So2	*Kosakonia sp.*	ND		ND		136.25	a	39.13	c	365.25	a	540.63
ATCC 27853	*P. aeruginosa*	ND		In excess of 300 ppm (661.57)	a	121.61	ab	ND		Below detection limit (24.87)	d	808.05

Values in parenthesis are estimated based on the calibration curve
ND= None Detected

Soybean Greenhouse Test

A soybean greenhouse trial was conducted to see if the phosphate solubilizing abilities and the plant growth promoting characteristics were beneficial to soybean growth. By utilizing insoluble P and by only applying 10% of the Hoagland phosphorus fertilizer rate, we were able to assess if the isolates could help the plant to overcome the phosphate stress in vivo. The only treatment that received the full Hoagland phosphate fertilizer level was the highP treatment. Plant growth responses were based on plant biomass, root architecture, and phosphorus levels within the plant. Plant shoot and root biomass are shown in Figure 19. *Enterobacter* (Tr3R3) was the only isolate that had a statistically ($P \leq 0.05$) greater shoot biomass than the lowP treatment. Tr3R3 and Tc3So2 had numerically greater shoot masses than the highP treatment which received 90% more phosphate than the isolates. Two isolates (Tr3R3 and Tc3So2) had statistically ($P \leq 0.05$) great root mass than the lowP, highP, and *P. aeruginosa* isolate. All 7 of the isolates had statistically ($P \leq 0.05$) greater root biomasses than the lowP control.

The phosphorus level within the plant and shoot portions were determined using the vanadomolybdophosphoric acid colorimetric method. Utilizing this method, we were able to assess if the PSB isolates were able to make the insoluble P source available to the soybean plant in vivo. Figure 20 shows both the shoot and root phosphorus levels within the plant. There was no statistical difference in the shoot P levels, however similar to the shoot biomass values in Figure 19, Tr3R3 and Tc3So2 had the greatest numerical values. The root phosphorus levels are also shown in Figure 20, indicating that the lowP treatment did indeed have the lowest P level within the roots. The lowP treatment had a statistically ($P \leq 0.05$) lower level of P than Tr3R3, Tc3So2, Tr1R2, and M2R1. Once again Tr3R3 and Tc3So2 had the highest numerical levels of P which is representative of the isolates with the greatest root biomass as well.

The additional soybean greenhouse trial produced root architecture values as well, indicated in Table 29. Additional information from that greenhouse trial can be found in the supplemental material. The same parameters and treatments were used in this trial, with the exception of additional replications. Table 29 shows root surface area and root volume parameters from the architecture scans. The root surface area data shows that all treatments, with the exception of M3So1, highP, and TrSH4, were able to increase the root area compared to the lowP treatment. Table 29 also shows that Tr3R3, M2R1, Tc2So2-1, Tr1R2, and *P. aeruginosa* had statistically ($P \leq 0.05$) greater root volume than the lowP treatment. For both the root surface area and root volume Tr3R3 had the largest values.

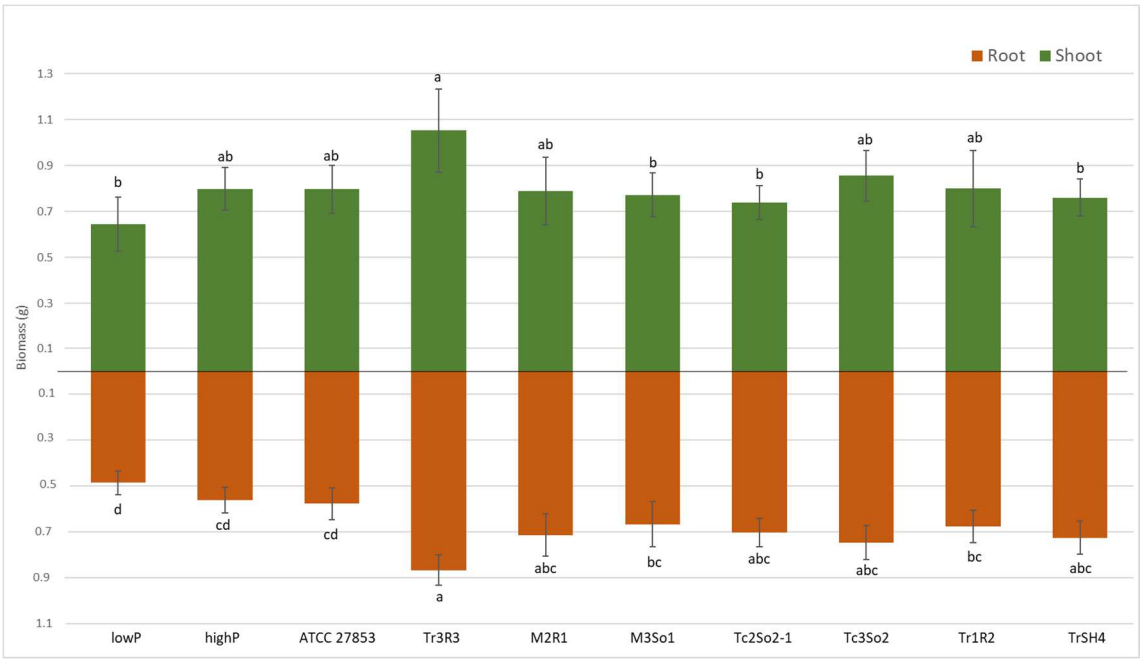

Figure 19. Soybean shoot and root biomass after 1.5 months of growth in the greenhouse. Shoot biomass is indicated in green and root biomass is indicated in orange.

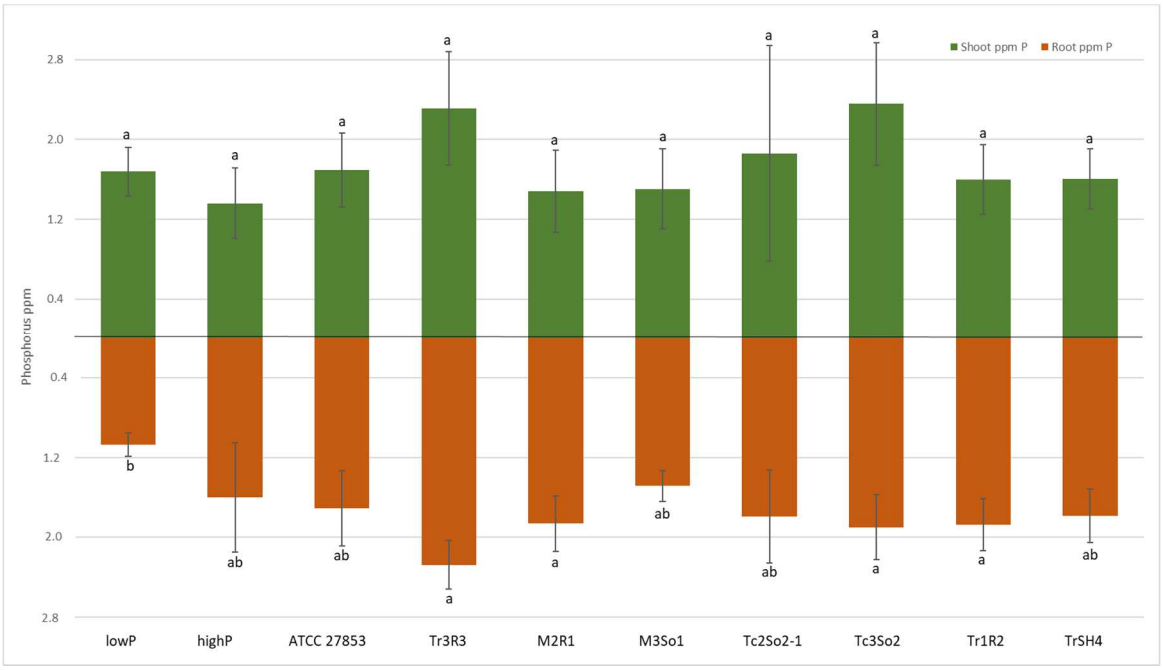

Figure 20. Phosphorus levels in the shoot and root of soybeans after 1.5 months of growth in the greenhouse. Shoot P (ppm) is indicated in green and root P (ppm) is indicated in orange.

Table 29. Root architecture measurements, such as root surface area (cm2) and root volume (cm3), from the second soybean greenhouse trial, determined by the WinRhizo scanning program.

Treatment	Root Surface Area (cm^2)	Root Volume (cm^3)
LowP	155.79 b	1.69 b
HighP	216.41 ab	2.54 ab
ATCC 27853	229.18 a	2.84 a
Tr3R3	268.75 a	3.20 a
M2R1	240.14 a	2.85 a
M3So1	217.91 ab	2.50 ab
Tc2So2-1	243.91 a	2.90 a
Tc3So2	228.80 a	2.59 ab
Tr1R2	233.67 a	2.95 a
TrSH4	208.84 ab	2.39 ab

Discussion

The majority of the PSB isolates were cultured from the loose root soil. To assess the phylogeny of the subset of the isolates, 16S rRNA sequences were determined for 21 of the isolates. Based on the 16S rRNA, most of the isolated bacteria were from the *Pantoea* genus, with 42.9% of the 21 isolates being that genus. The down selection of the 7 isolates were based on a selection of variation in both phylogeny and the phosphate solubilizing index. In addition to the 7 isolates, we utilized *Pseudomonas aeruginosa* ATCC 27853 as a positive control, due to the P-solubilizing abilities of *P. aeruginosa* in literature (Buch, Archana, & Kumar, 2008; Kothamasi, Kothamasi, Bhattacharyya, Kuhad, & Babu, 2006; Nautiyal, 1999). It is recorded that *P. aeruginosa* ATCC 27853 had strong growth on Pikovskayas agar and was positive for clearing zones around the growth (HiMedia, 2015). These 8 isolates were tested for numerous characteristics related to phosphate solubilizing abilities and plant growth promoting

characteristics to determine if any of the isolates would potentially be suitable as a bioinoculant.

First the isolates were characterized using several plant growth promoting assays. Motility of soil and endophytic bacteria is a key advantage to colonize the plant and move to nutrients within the soil (Bohm, Hurek, & Reinhold-Hurek, 2007). *P. aeruginosa* was slow to grow within the media, however there has been reports of this due to some strains of *P. aeruginosa* being strictly aerobic (Armitage & Evans, 1983). The three isolates that were not motile were M2R1, Tc2So2-1, and Tr1R2. This is further backed up by the full genome annotation of M2R1, there are no motile genes based on the RAST motile subsystem features. Considering, Tr1R2 and Tc2So2-1, the two *Bacillus* species, were not motile and the only isolates that did not solubilize any of the P sources of the quantification assay, these isolates may not be as efficient in colonizing the insoluble surface area as the other isolates. Similarly, Tr1R2 and Tc2So2-1 were the only isolates that were not able to grow on nitrogen free media or increased the pH of the agar. Both Tr3R3 and M2R1 do not possess *nif* genes but based on the RAST annotation, they do have 36 reported nitrogen metabolism subsystem features. Indole-3-acetic acid biosynthesis is a common component of selecting an effective bioinoculant, due to the positive impact on plant growth (Tao et al., 2008). The three isolates Tc2So2-1, Tr1R2, and M2R1 had the highest levels of IAA production, and Tc2So2-1 had a statistically ($P \leq 0.05$) greater amount of IAA produced than all the other isolates. And finally, fungal suppression is an important feature to look for in a bioinoculant due to the detrimental effects it has on food production worldwide and the negative effects of fungicides on

human and environmental health (Lorenz, 2017; Wegulo et al., 2015). The only two isolates that suppress all four of the fungal species were the two *Bacillus* strains Tc2So2-1 and Tr1R2. The isolates that caused the lowest level of fungal suppression were T3So2 and TrSH4.

Phosphate solubilizing biofertilizers are the fastest growing sector of the bioinoculant industry, and this is due to the plant's critical need for P as well as the finite nature of the resource (Grand, 2018). All 8 isolates were screened for phytase and acid phosphatase production, as well for their ability to solubilize inorganic P. When comparing the results of the phytase and acid phosphatase plate assays, Tr3R3 and Tr1R2 did not produce phytase but did produce acid phosphatases, whereas *P. aeruginosa* did produce phytase but not acid phosphatase. In fact, *P. aeruginosa* mineralized a substantially greater amount of phytic acid than all other isolates. The plate assays show that M2R1 was able to mineralize a moderate amount of phytic acid and was a strong mineralizer of phenolphthalein diphosphate, when comparing these findings to the annotated genome M2R1 does not possess the phytase gene but does contain an acid phosphatase gene. Similarly, when comparing Tr3R3's plate assay results that was negative for phytic acid, but positive for phenolphthalein diphosphate mineralization, Tr3R3 in fact does not possess the phytase gene and does contain an acid phosphatase gene, correlating to its functional abilities. The qualitative inorganic P assay was conducted on PVK agar, showing *P. aeruginosa* with one of the largest clearing zones, substantiating the selection of that species as the positive control. The only other isolate that had a phosphate solubilizing index as great as P. *aeruginosa* was

M3So1. However, when quantifying the amount of phosphate made soluble by the isolates, these results did not correspond to the P-solubilizing index, this is similar to other findings. Previously, it has been shown that bacteria that do not create a clearing zone on agar plates can solubilize inorganic phosphate in broth assays, and vise-versa (Nautiyal, 1999). Isolates Tc3So2, Tr3R3, and M2R1 solubilized significantly ($P \leq 0.05$) more phosphate than the other isolates. The two *Bacillus* species, Tc2So2-1 and Tr1R2, did not solubilize any phosphate compared to the control level. Based on these findings Tr3R3 and M2R1 were selected for whole genomic sequencing.

The full genomes made it possible to look for key phosphate solubilizing genes as well as additional plant growth promoting genes. Tr3R3 and M2R1 possess the same genes relating to phosphate solubilizing and metabolism based on the Galaxy annotation. A lot of the genetic work on the inorganic solubilizing organic acids has been focused on gluconic acid, it has been difficult to find a homologous gene that could be used to help identify a universal selector of a PSB based on organic acid production (Zheng, Hao, et al., 2017). With that in mind we were only able to look for the gluconic acid producing genes. Glucose dehydrogenase (*gcd*) and gluconate dehydrogenase (*gad*) are key genes in that system, however Tr3R3 and M2R1 possessed the *gcd* gene but not the g*ad* gene. The genes typically identified on the organophosphate mineralization and metabolism side are the two-component regulatory system (p*hoR-phoP* or *phoR-phoB*), the phosphate specific transporters (*pstS*CAB), regulation of the Pho regulon (*phoU*), storage of inorganic P (*ppk*), and several exoenzymes (*phoA, phoD, phyC, glpQ, ushA, aphA)*. Both Tr3R2 and M2R1 contain all the above mentioned genes, with the exception

of the phospholipases (*phoD*) and phytase (*phyC*). RAST SEED Viewer was also used to identify potential additional subsystem features used in plant growth promoting abilities. As previously stated M2R1 does not possess motility genes, whereas Tr3R3 contains 15 subsystem features. Both species possess 17 invasion and intracellular resistance features, putatively used by endophytic bacteria (Tharek, Sim, Khairuddin, Ghazali, & Najimudin, 2017). Tr3R3 has 27 subsystem feature counts for siderophores and M2R1 has 29. Siderophores are beneficial in acquiring iron but have also been related to the ability to solubilize phosphate (Sharma et al., 2013). Fourteen Tr3R3 and 9 M2R1 subsystem features of quorum sensing and biofilm formation were identified, helping the bacteria to attach to plants surfaces. Auxin, a plant growth hormone produced by some microbes can help increase plant growth (Prikryl, Vancura, & Wurst, 1985), and in both species 5 auxin features were identified. As stated before the two species do not possess the majority of the *nif* genes, they only contain the pyruvate-flavodoxin oxidoreductase (*nifJ*) gene. However, they both contain 36 nitrogen metabolism subsystem features.

The organic acids formic, malic, lactic, citric, and succinic were measured to determine the mechanism of solubilizing the inorganic insoluble phosphorus. Based on Table 28 we can conclude, based on these 8 isolates, that formic acid was not a dominant acid in the solubilizing ability due to the very low levels. It appears that *P. aeruginosa* produced a substantially greater amount of malic acid compared to all the other isolates and acids, however it needs to be noted that the peaks for these samples were much wider than the other analyzed peaks, producing a seemingly higher

concentration. Lactic acid had a range of 6.26 ppm to 210.75 ppm, and the two highest values were produced by M3So1 and Tc3So2. Citric was produced by Tr3R3, TrSH4, T2R1, and Tc2So2; however, it was significantly highest in the Tr3R3 isolate. And finally, the most frequently produced acid and the highest concentration was succinic acid. Tr3R3 and Tc3So2 had significantly higher levels of succinic acid than the other isolates. Tr3R3 and Tc3So2 solubilized the greatest amount of phosphate in the P-solubilizing quantification assay, and they also produced the greatest amount of succinic acid. Also, Tr1R2 and Tc2So2-1 were the only isolates that did not solubilizing any phosphate in the quantification assay compared to the control, and they were also the only isolates that did not produce succinic acid. With these two findings in mind, we would conclude that, from the five acids measured, succinic acid has the greatest effect on solubilizing phosphate. The total acid concentration is also a good indicator that the organic acids are the key P-solubilizing mechanisms. Tr3R3 and Tc3So2 produced the highest total concentration of acid, only after *P*. *aeruginosa,* potentially due to the width of the peaks. Tr1R2 and Tc2So2-1 produced the lowest concentration of acids, which directly corresponds to the amount of phosphate each isolate was able to solubilize. Also, Tr1R2 and Tc2So2-1 had the highest pH of the isolates, corresponding to the two isolates producing substantially lower levels of organic acids that the other isolates.

To determine which plant growth promoting and phosphate solubilizing effects were most beneficial in vivo, a soybean greenhouse experiment was conducted. Due to the plants receiving insoluble phosphorus in the potting medium and only 10% of the Hoagland fertilizer levels, the bacteria that quantitively solubilized the greatest amount

of phosphate, performed the best in the greenhouse trial. Tr3R3 and Tc3So2 had statistically greater root biomass than both the highP and lowP levels, and Tr3R3 had a significantly greater shoot biomass than the lowP treatment. These results correspond to the in vitro quantitative phosphate test. Most likely indicating the two isolates are able to release organic acids or other mechanisms that solubilizing inorganic phosphorus forms in the soil, making the P available for the plant to utilize. The additional greenhouse trial provided in the supplemental data, also shows Tr3R3 to have a statistically greater root biomass than the highP and lowP. The root architecture parameters from this trial, shown in Table 29, indicate Tr3R3 to have the greatest root surface area and root volume values compared to all the treatments. Following Tr3R3 for root surface area are Tc2So2-1 and M2R1 which had the two highest IAA biosynthesis values. IAA is reported to impact lateral and primary roots and root hair growth and formation (Uggla et al., 1996). These isolates, Tc2So2-1 and M2R1, potentially increased root surface area and volume compared to the lowP control due to their significantly high IAA production. The ranking of the isolates for the root phosphate levels corresponds to the ranking of isolate for the root biomass values. Tr3R3 seems to have the greatest impact on the soybean growth and phosphate levels, and when comparing that to the screening assays and genomic information, we conclude that the benefit is most likely due to the high levels of phosphorus it is able to solubilize. Tr3R3, Tc3So2, and M2R1 all solubilized a statistically similar amount of phosphorus, however Tr3R3 and Tc3So2 seemed to have a greater impact on plant growth and plant phosphate levels. This potentially is due to the fact that Tr3R3 and

Tc3So2 are motile where M2R1 is not, making it more difficult to access the same amount of nutrients and colonize the plant. And finally, due to Tr3R3 and Tc3So2 producing the highest levels of succinic acid which seemed to have the greatest impact of P solubilization. Overall it would appear that T3R3 and Tc3So2 are the best candidates for bioinoculants.

These results are validated by others research showing that *Enterobacter cloacae* is an optimum phosphate solubilizer and plant growth promoting bacteria. It was found that *E. cloacae* was a strong P-solubilizing abilities and increase wheat growth, chlorophyll levels, and yield (Borham, Belal, Metwaly, & El-Gremy, 2017). Additionally, when a strain of *E. cloacae* was applied to sugarcane and low levels of P fertilizer were applied, the *E. cloacae* strain improved P uptake into the plant, whereas the non-inoculated plants had a diminishing P uptake effect over time (Safirzadeh, Chorom, & Enayatizamir, 2019).

Conclusions

Of the 8 isolates tested, *Enterobacter cloacae* (Tr3R3) seemed to have the greatest potential as a bioinoculant. This isolate is motile, was able to grow on nitrogen free media, have putative plant growth promoting genes such as siderophore production, biofilm and quorum sensing, auxin biosynthesis, and nitrogen metabolism genes, it produced acid phosphatases, and is a strong phosphate solubilizers. Tr3R3 was able to solubilize the greatest amount of inorganic phosphate from the 8 isolates tested. The organic acid levels produced by the isolate appeared to be the mechanism used to

solubilize the phosphate. The key organic acid in the solubilizing process was succinic acid, and Tr3R3 produced the greatest amount of succinic acid. The isolates proved to be beneficial to the growth of soybean plants, and the assimilation of phosphate into the plant. We conclude that *Enterobacter cloacae* (Tr3R3) is an optimal and promising bioinoculants.

Conclusion

Due to the need for a safe and abundant food supply, researchers have strived to find new ways of increasing crop producing while sustaining the land we have for production purposes. Bioinoculants have many beneficial characteristics that have been proven to increase plant growth while maintaining the arable label. Due to the increases in culture-independent techniques and genomic analysis, a large library of potential bioinoculants have been growing over the years.

We identified and characterized four novel bacteria in the Oxalobacteraceae family; *Pseudoherbaspirillum sperare, gen. nov., sp. nov.* OM1, *Massilia arenosa, sp. nov.* MC02, *Massilia hortus, sp. nov.* ONC3, and *Duganella callidus, sp. nov.* DN04. These were identified as novel bacteria based on phenotypic and genomic analysis. These isolates can potentially be utilized as bioinoculants due to their culturing locations from agricultural soils as well as their beneficial genomic information. Some of the putative plant growth promoting genes that were identified were; nitrate reductase, urease, phosphatase, biotin production, decomposition on hydrogen peroxide, biofilm biosynthesis, and intracellular invasion genes which suggests these are potential endophytic bacteria.

Phosphate solubilizing capabilities was more specifically investigated by isolated 70 bacteria from maize plants. All the isolates were screened for P-solubilizing abilities, and further testing was conducted on 8 isolates. Several of the 8 cultures were able to grow on nitrogen free media, produce IAA from tryptophan, and suppress the growth of fungal pathogens, suggesting these bacteria were potentially causing plant growth

promoting benefits in the rhizosphere and within the maize plant. When quantifying the amount of inorganic P these isolates were able to solubilize, Tc3So2, Tr3R3, and M2R1 solubilized the greatest amounts (79.2, 75.68, and 72.16 mg P/L respectively), and Tr1R2 and Tc2So2-1 did not solubilize any phosphate. When comparing that to the HPLC organic acid assay, Tc3So2 and Tr3R3 produced the greatest amount of succinic acid (365.25 and 384.49 ppm respectively) and Tr1R2 and Tc2So2-1 did not produce any succinic acid, concluding that succinic was the key organic acid utilized in the solubilization process. These benefits correlated to an increase in plant biomass, root architecture and P uptake concentrations. Tr3R3 had a statistically greater root biomass than the high and low phosphate controls, a statistically greater shoot biomass than the low phosphate control, and a statistically great root surface area and volume than the low phosphate control. Tc3So2 had a statistically greater root biomass than the low phosphate control. Tr3R3 and Tc3So2 both had a statistically greater phosphate level within the root than the low phosphate control. These results show that when a plant is stressed with low levels of available phosphorus, PSB are able to solubilize the P in the soil and make it available to the plant.

By isolating and characterizing novel bacteria from agricultural soils and testing phosphate solubilizing bacteria, we have seen that rhizosphere and endophytic bacteria have a great capacity to increase plant performance. We propose the use of plant growth promoting bacteria and phosphate solubilizing bacteria as bioinoculants to increase food production in a sustainable way. This can be further studied by large scale field trials as well as genomic analyzing. There has been little genomic research done on

the different organic acids produced by PSB. The more this area is elucidated, the faster the screening process will be for plant growth promoting and PSB bacteria.

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