HOW BENEFICIAL *PSEUDOMONAS FLUORESCENS* PROTECT *ARABIDOPSIS THALIANA* FROM AN OPPORTUNISTIC PATHOGEN

by

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Abstract

Plants form commensal associations with soil microorganisms, creating a root microbiome that provides benefits to the host including protection against pathogens. While bacteria can inhibit pathogens through production of antimicrobial compounds *in vitro*, it is largely unknown how microbiota contribute to pathogen protection *in planta*. I used a gnotobiotic model system consisting of *Arabidopsis thaliana*, and an opportunistic pathogen *Pseudomonas* sp. N2C3, to identify mechanisms that determine the outcome of plant-pathogen-microbiome interactions in the rhizosphere. I screened 25 phylogenetically diverse *Pseudomonas* strains for their ability to protect against N2C3 and found that commensal strains closely related to N2C3 were more likely to protect against pathogenesis. I used a comparative genomics approach to identify unique genes in the protective strains that revealed no genes that correlate with protection, suggesting that variable regulation of components of the core *Pseudomonas* genome may contribute to pathogen protection. I found that commensal colonization level was highly predictive of protection and so tested deletions of genes previously shown to be required for *Arabidopsis* rhizosphere colonization. I identified a response regulator *colR* that is required for *Pseudomonas* protection against N2C3 and fitness in competition with N2C3 indicating that competitive exclusion may contribute to pathogen protection. I found that *Pseudomonas* sp. WCS365 also protects against the agricultural pathogen *Pseudomonas fuscovaginae* SE-1, the causal agent of bacterial sheath brown rot of rice. This work establishes a gnotobiotic model to uncover mechanisms by which members of the microbiome can protect hosts from pathogens and informs our understanding of the use of beneficial strains for microbiome engineering in dysbiotic soil systems.

Lay Summary

The area surrounding plant roots can house a diverse and abundant community of bacteria and other microbes called the microbiome. Though many bacterial strains in the genus *Pseudomonas* coexist peacefully with plants, some can cause disease. As members of the microbiome can protect against pathogens, I tested whether beneficial *Pseudomonas* strains could protect against those that cause disease. I found that *Pseudomonas* strains differed in their ability to protect plants from a *Pseudomonas* pathogen, and that most of the protective strains were closely-related to the pathogen. I observed that protection was correlated with bacterial root colonization, which led me to discover three colonization genes necessary for protection. I also observed that protective strains could protect against more serious agricultural pathogens including the bacterium that causes rice sheath brown rot. This suggests that members of the microbiome might be useful for combatting a broad range of bacterial pathogens.

Preface

The works included in this thesis were conducted under the supervision of Dr. Cara H. Haney, at the University of British Columbia, Vancouver. This thesis was written by me, and edited by Dr. Cara Haney.

Experimental methods were approved by the University of British Columbia's Office of Research Sciences Biosafety Committee [certificate #B21-0088]. I designed all experiments, with support from Dr. Cara Haney, and conducted all experiments and data analyses, with the following exceptions.

- The initial idea for screening beneficial *Pseudomonas* strains for their ability to protect against N2C3 was conceived by Dr. Ryan Melnyk. Sarzana Hossain performed a preliminary screen of 12 *Pseudomonas* strains, at a 1:1 ratio. However, data from her screen was not included in this thesis, and all follow-up experiments were conceived and designed by me.
- I designed and performed initial screens of all *Pseudomonas* strains and mutants indicated in this thesis, to test for protection at 5:1, 1:1, and 1:5 ratios. Additional replicates were performed with assistance from UBC undergraduate Kitty Martens under my supervision.

Chapter 1 and Chapter 3 contains a partial reproduction from the published review written by myself and edited by Dr. Haney (© Nicole R. Wang and Cara H. Haney):

> Wang NR, Haney CH. 2020. Harnessing the genetic potential of the plant microbiome. Biochem (Lond) 42: 20-25. doi: https://doi.org/10.1042/BIO20200042

The abstract and Chapter 2 is a partial reproduction from a submitted paper, which is also available as a Preprint:

Wang NR, Melnyk RA, Wiesmann CL, Hossain SS, Chi M, Martens K, Haney CH. 2021. Commensal *Pseudomonas fluorescens* protect *Arabidopsis* from closely-related *Pseudomonas* pathogens in a colonization-dependent manner. *bioRxiv*. doi: https://doi.org/10.1101/2021.09.02.458786

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Melnyk RA, Hossain SS, Haney CH. 2019. Convergent gain and loss of genomic islands drive lifestyle changes in plant-associated *Pseudomonas*. ISME J 13:1575–1588. doi: https://doi.org/10.1038/s41396-019-0372-5

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List of Symbols

β	beta
Δ	deletion
°	degrees
γ	gamma
λ	lambda
μ	micro

List of Abbreviations

16S rRNA	16S ribosomal ribonucleic acid
ACC	Aminocyclopropane-1-carboxylic acid
AHL	N-acylhomoserine lactone
ANOVA	Analysis of variance
BCM clade	Brassicacearum, Corrugata, Mediterranea clade
bp	Base pairs
Col-0	Columbia
CFU	Colony forming unit
DAPG	2,4-Diacetylphloroglucinol
DNA	Deoxyribonucleic acid
Gm	Gentamycin
GWAS	Genome-wide association study
HCN	Hydrogen cyanide
HSD	Honestly significant difference
IAA	Indole-3-acetic acid
ISR	Induced systemic resistance
KB	King's B
LB	Lysogeny broth
LPQ island	Lipopeptide/quorum sensing island
LPS	Lipopolysaccharide
MAMP	Microbe-associated molecular pattern
MES	2-(N-morpholino)ethanesulfonic acid

MS	Murashige and Skoog
N2C3	*Pseudomonas* sp. FW300-N2C3
OD_{600}	Optical density, measured at 600 nm
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PGPR	Plant growth promoting rhizobacteria
Pseudo.	*Pseudomonas*
PTI	Pattern-triggered immunity
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
SD	Standard deviation
SNP	Single nucleotide polymorphism
sp.	Species
spp.	Species pluralis
SYP	Syringopeptin
SYR	Syringomycin
T3SS	Type III secretion system
Tn-seq	Transposon insertion sequencing
WCS365	*Pseudomonas* sp. WCS365
WT	Wildtype
X-gal	5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside

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I would like to sincerely thank my supervisor, Dr. Cara Haney, for her mentorship, compassion, and endless ability to generate ideas, making my experience in her lab extremely enriching. I also extend my gratitude to my committee members, Dr. Jim Kronstad, Dr. Steven Hallam, and Dr. Xin Li, for providing constructive feedback and ideas for my project. I also thank the staff at UBC and my funding agencies including NSERC, Mitacs, and the George Weston Foundation. I am grateful to collaborators from the Noble Foundation Dr. Myoung-Hwan Chi and Dr. Kelly Craven for graciously providing several strains used in this study.

I would like to thank everyone who contributed to this work in even the smallest capacity: from creating deletion mutants, to engaging in helpful discussions, to streaking out a bunch of bacteria for me. These include Dr. Ryan Melnyk, Christina Wiesmann, Sarzana Hossain, Kitty Martens, Dr. David Thoms, Jessie Zhang, Frank Liu, Siyu Song, Yang Liu, and probably many others. I am grateful for all my lab members, past and present, for their help and support, both in and out of the lab. I am extremely thankful for the many hours we've spent together and for creating a welcoming laboratory environment. I would like to thank the friends I've made throughout my degree, including the members of the Haney Lab, Kronstad Lab, PRoTECT, and MIGSS, who allowed me to remain socially connected throughout a pandemic.

Special thanks to my parents, family, and friends, who provided me with endless support even when we were apart.

Dedication

To my grandmother, Boualy Wang.

Chapter 1: Introduction

1.1 Plants form microbiome associations that expand their genetic potential

Similar to animals, plants have evolved to form complex, beneficial relationships with the microorganisms in their surroundings. These plant-associated microorganisms can be found on a variety of tissues, and collectively make up the plant microbiome. Although the plant microbiome includes bacteria, fungi, archaea, protists, and viruses, the majority of research has focused on bacterial communities, and to a lesser degree, fungal communities (1). The diverse communities of bacteria and fungi in the plant microbiome can have beneficial impacts on their hosts, such as improving growth, even when faced with challenges caused by biotic and abiotic stresses. Taking advantage of the benefits of the plant microbiome in agricultural practices could help enhance disease resistance, improve tolerance to abiotic stresses, and help with nutrient uptake (2–4). Consequently, the presence of these beneficial members of the plant microbiome could lead to improved crop yields, while decreasing the amount of fertilizer and pesticides applied.

Plants have a variety of organs that can be inhabited by microorganisms (Figure 1.1A). The rhizosphere, which is the root surface and nearby nutrient-rich soil environment that is affected by secreted exudates, is generally the niche with the highest abundance and species diversity. Estimates are that up to 20-40% of plant photosynthate is secreted into the rhizosphere, making it one of the most nutrient-rich environments in the soil (5). Plant shoots and leaves (the phyllosphere) can also harbour microbes, though usually fewer organisms are able to survive in these nutrient-poor, and more exposed surfaces. A subset of specialized microorganisms can also colonize the inside of roots or shoots, known as the endosphere. The majority of microorganisms that form the plant microbiome originate from the surrounding soil or air (horizontal

transmission) with a small subset inherited from the parent generation through seeds (vertical transmission) (6).

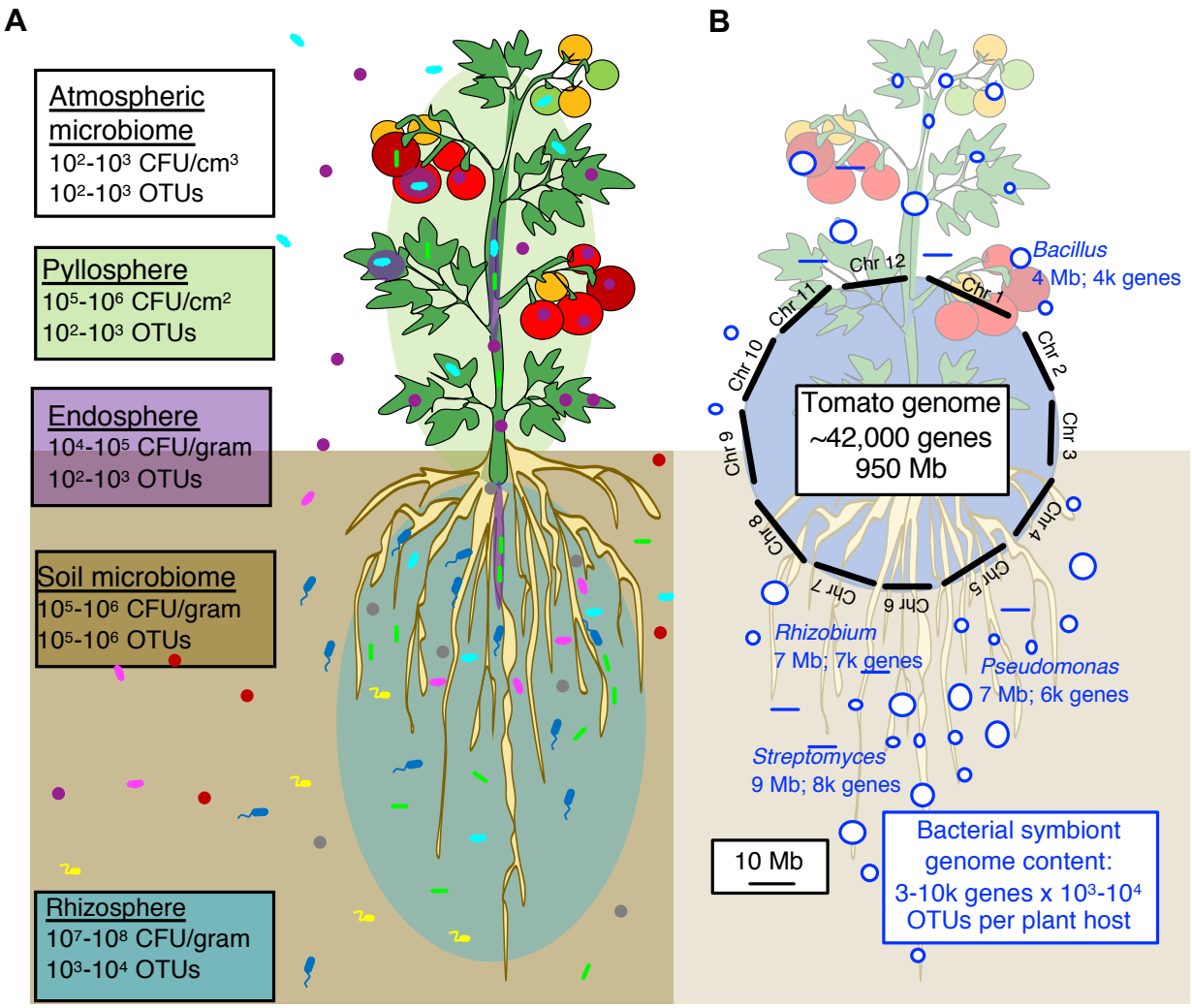

Figure 1.1 The plant microbiome expands the genetic potential of plants. (A) Plant niches for microbes include the phyllosphere, rhizosphere and endosphere, the majority of which are horizontally acquired from the soil or air. Estimates for the number of colony-forming units (CFUs) and operational taxonomic units (OTUs), a metric of bacterial species diversity, are provided for each biological niche. (B) Collectively, the plant microbiome expands the plant's potential to acquire nutrients, defend against pathogens, and maintain nutrient homeostasis. Plant genomes range from ~25,000-100,000 protein-encoding genes (ex. *Arabidopsis* has ~27,000 genes, tomato has ~42,000 genes, rice has ~40,000 genes, and wheat has ~100,000 genes). A single bacterial genome may encode 3,000-8,000 genes and a typical plant supports 1,000-10,000 bacterial OTUs. Although many bacterial strains encode genes with overlapping functions in their core genomes, strains within a single I may have up to 25% variation in their accessory genomes (7).

The sheer abundance and diversity of microorganisms in the plant microbiome allows it to provide a large reservoir of genes that can significantly impact plant fitness. The number of genes present in members of the plant microbiome can greatly outnumber the genes in the plant genome itself (Figure 1.1B). Plant genomes range from around 25,000 genes to up to 100,000 genes for the largest genomes like wheat. Bacteria may range from 3,000-8,000 genes per genome, but a typical plant supports 1,000-10,000 bacterial OTUs. Although many bacterial strains encode genes with overlapping functions in their core genomes, strains within a single I may have up to 25% variation in their accessory genomes (7). While many bacterial taxa have shared genetic content (their "core genomes"), the full collection of genes in a single genus (the pangenome) may exceed 15,000 genes (7), indicating that the thousands of microbes associated with plants likely greatly exceeds the genetic potential of the plant. Since plants can shape their rhizosphere microbiome in response to stresses, plants can potentially harness the large functional diversity in their microbiomes for better adaptation to environmental conditions. Therefore, the genetic potential of the plant-associated microbiome should be taken into account when assessing the total genetic potential of a plant.

1.2 The plant microbiome shifts in response to growth conditions

The composition of a plant's microbiome is dynamic, and can change over the course of a plant's lifetime (8, 9). Plant developmental stage, genotype, and growth conditions can correlate with shifts in plant microbiome composition (Figure 1.2) (10, 11).

For plants and other organisms, the core microbiome of a species is defined as the microbial taxa that associate as a result of evolutionary selection, though for some plants, the exact strains may vary between soils due to functional redundancy (6). In contrast, the accessory

microbiome consists of the microorganisms that help plants adapt to their specific environmental condition (12). Additionally, rare taxa have been shown to have a larger contribution to microbiome functions than expected based on their abundance (13, 14).

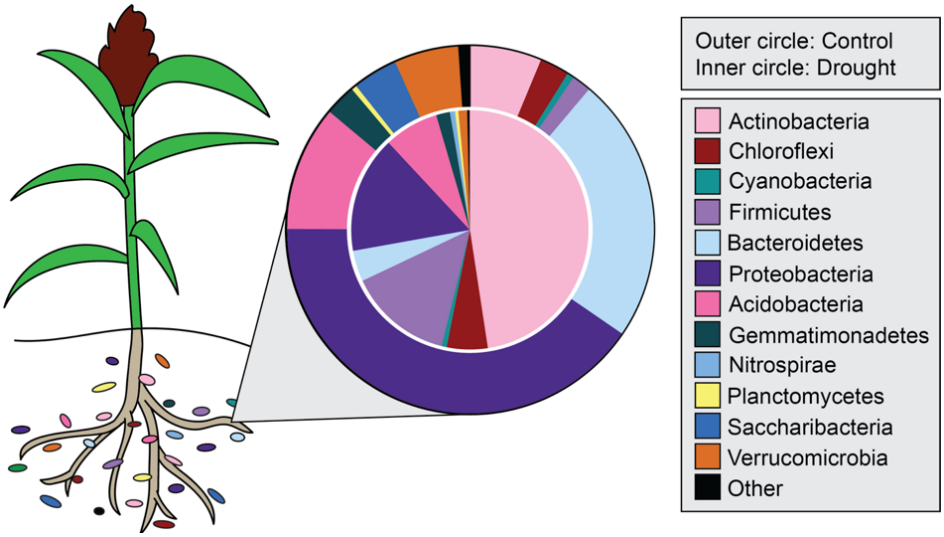

Figure 1.2 The microbiome composition changes in response to drought stress. Shown is the relative abundance of the most abundant bacterial phyla in the sorghum rhizosphere microbiome (outer circle) and the changes in response to drought (inner circle). Using 16S rRNA amplicon sequencing data from a study performed by Xu et al. (10), the bacterial composition of the rhizosphere microbiome of pre-flowering sorghum plants was compared under 8 weeks of well-watered or drought conditions.

1.3 Microbes can mediate benefits to plant health.

Plants actively form beneficial relationships with microorganisms to help them grow and respond to challenges in their local environment. Beneficial bacteria that colonize the roots and rhizosphere are often called plant growth-promoting rhizobacteria (PGPR) because of their positive effect on plant health. Bacteria in the phyllosphere can induce plant immune responses and are known to play roles in pathogen protection. Plants can also form highly species-specific mutualistic interactions with microorganisms in the endosphere, such as nodule-forming bacteria and arbuscular mycorrhizal fungi, which can improve plant access to essential nutrients.

Associating with microorganisms can provide plants with novel genes and functions, such as nutrient uptake and antimicrobial production. Plants require nitrogen to survive but do not have genes that encode nitrogenase to convert atmospheric nitrogen into usable forms. A subset of plants has evolved symbiotic relations with nitrogen-fixing bacteria. For example, legumes can form nodules filled with nitrogen-fixing bacteria that provide the plant with a reliable source of nitrogen. While symbiotic nitrogen fixation has long been viewed as legume-specific, plant-associated microbiomes are consistently enriched in bacteria that have genes that encode for nitrogen-fixation enzymes (15). This suggests that many plants may receive a benefit from providing an ecological niche for microbial nitrogen fixation. Similarly, soil microorganisms can play an important role in increasing the availability of other essential nutrients to plants. Arbuscular mycorrhizal fungi can transport solubilized phosphorus and zinc into plant cells, whereas many PGPR can produce siderophores that increase iron availability for the plant (16).

Members of the plant microbiome can also provide plants with an adaptive advantage by modulating existing plant functions. Many beneficial microorganisms are capable of producing or degrading phytohormones, impacting plant hormone homeostasis with consequences for growth and development. For example, PGPR that synthesize auxin induce lateral root production, increasing the plant's ability to take up water and nutrients (5). Some PGPR can produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase, an enzyme that blocks plant ethylene biosynthesis in response to stress, and therefore prevents root stunting (17).

1.4 Harnessing the microbiome for agricultural improvement

Many conventional agricultural practices do not consider the positive impact of microorganisms on plant growth. The majority of beneficial microorganisms that form the plant microbiome are recruited from the soil. However, current agricultural practices use a mixture of strategies that can drastically modify or harm the natural soil microbiota, impacting the diversity of microorganisms used to build the plant microbiome, and creating dysbiotic soil systems. These strategies can include the use of pesticides, fertilizers, or soil fumigation. In aeroponic or hydroponic systems, plants are grown without soil, losing access to a large source of beneficial microorganisms and potentially leaving them vulnerable to opportunistic pathogens. Therefore, enhancing the plant microbiome has a great potential to improve crop yields, especially as growing populations and climate change raise concerns for food security.

Gaps in current agricultural practices could potentially be filled by adding beneficial microbes into dysbiotic agriculture soils, thereby reintroducing beneficial genes to the plant. Researchers have discovered many PGPR that provide plants with an advantage under stressful conditions (2). In controlled lab and greenhouse experiments, the plant microbiome can be manipulated by introducing desirable PGPR strains, subsequently leading to improved plant growth under stress (3). However, there has been limited success when testing PGPR inoculants in agricultural soils. This may be because the introduced microbe is unable to outcompete existing microbes, adapt to the local soil environment, successfully colonize the plant, or express the beneficial genes. The outcomes of these trials are highly dependent on the soil type, environmental conditions, and plant genotype, so even if a PGPR is successful in one field, it may not work in another.

Although the presence of certain members of the microbiome improves plant health, many of the genetic mechanisms for these processes are still unknown. In order to fully harness the genetic potential of the microbiome, it is essential to identify the underlying genetic and molecular mechanisms to translate this research for agricultural applications (Figure 1.3). For instance, having genetic markers for specific traits could help with screening for desirable PGPR that are adapted to a specific soil or plant. Alternatively, known beneficial microbial molecules can be purified and added to plants in lieu of conventional pesticides or fertilizers. This is currently done with Bt toxin, an insecticidal compound produced by the plant-associated microbe *Bacillus thuringiensis.* Knowing the beneficial microbial genes can facilitate genetic engineering approaches to introduce beneficial microbial traits into plants. Finally, understanding what allows individual microbes to colonize specific plants, and how plants shape their associated communities, can guide breeding strategies.

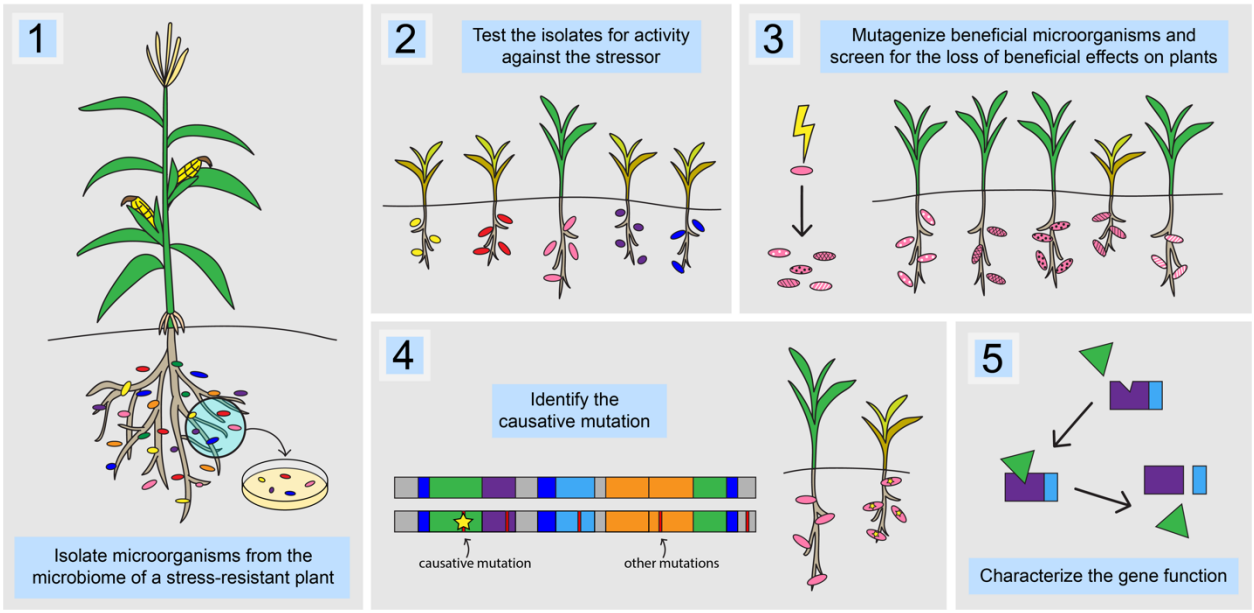

Figure 1.3 The process of identifying genetic mechanisms behind plant growth-promoting traits in plant-associated microorganisms. Images outline an experimental process leading to the discovery of ACC deaminase, an enzyme that can promote plant growth under salinity stress. (1) Rhizobacteria are isolated from healthy maize growing in saline soil. (2) Each isolated bacterium is inoculated individually into the rhizosphere of maize in saline soils, to identify the strains that promote growth. (3) The beneficial bacterial strain (pink) is chemically mutagenized. The mutagenized variants (pink, patterned) are individually screened for a loss of protection against salt stress. (4) The mutagenized strain that had a loss of beneficial effects is sequenced, and its mutations are mapped to the wildtype genome. The causative mutation is confirmed by testing gene deletion mutants under salt stress. (5) The gene encodes ACC deaminase (green) that cleaves ACC (purple and blue) into ammonia and α-ketobutyrate, upon binding (17).

1.5 Rhizosphere colonization is essential for microbiota to benefit their plant hosts

Rhizosphere colonization is important to consider prior to using bacteria for microbiome engineering, and is essential for lab-based discoveries to be translated into field settings. Bacteria introduced into the rhizosphere must be able successfully colonize the plant roots in competition with existing soil microbes, and ideally withstand differences in environmental conditions, soil type, and plant genotype. Understanding the mechanisms that allow microbes to colonize plants

can help select or engineer bacteria that have greater potential to be maintained in the rhizosphere, and provide the intended effects.

The ability of microbes to colonize the rhizosphere depends on many factors, such as the ability to use carbon from root exudates, amino acid biosynthesis, evasion of the plant immune responses, motility, chemotaxis, biofilm formation, and cell wall composition (18–22). Plant genetics also shapes microbial ability to colonize plant roots. For example, plant immune signalling via the hormone salicylic acid can modulate the microbiome composition by impacting the colonization of specific bacterial families (18). Understanding both the microbial factors that determine rhizosphere competence, and the plant genetic factors that shape the rhizosphere community, are critical to the use of microbiota to improve plant health in agriculture.

Genetic screening has been used to identify genes important for *Pseudomonas* to colonize the *Arabidopsis* rhizosphere. A transposon mutant screen found that disruption or removal of the O-antigen component of lipopolysaccharides (LPS) caused reductions in potato and tomato root colonization (21). Mutations in a *colR*/*colS* two-component system caused decreased outer membrane permeability, likely impacting uptake of root exudate nutrients and leading to a colonization defect (23). A transposon mutagenesis screen followed by next generation sequencing (Tn-seq) was conducted on *Pseudomonas simiae* WCS417r, identifying 358 genes within which mutations caused a significant increase or decrease in root colonization (24). These included genes involved in flagella biosynthesis, carbohydrate metabolism, and cell wall biogenesis (24). A Tn-seq screen performed on *Pseudomonas* sp. WCS365 identified 231 genes required for fitness including genes that improved *Arabidopsis* rhizosphere colonization through the evasion of plant defenses (25). These included a phosphodiesterase and a putrescine

aminotransferase that affect biofilm formation (25). Collectively these genetic approaches have revealed genes and mechanisms that are important for bacteria to colonize the plant rhizosphere.

1.6 Mechanisms by which microbiota protect against plant pathogens

Plant pathogens can detrimentally impact crop yields, contributing to up to 20-30% of global agricultural crop loss (26). However, through changes in root exudate composition upon exposure to a foliar pathogens, plants can shift their rhizosphere microbiomes, which can lead to formation of suppressive soils over many generations by the buildup of soil microbiota that can protect against the pathogen (9, 27, 28). Some bacteria, including *Pseudomonas* strains, are associated with suppressive soils due to their accumulation over successive generations and antifungal activity (28, 29). This has led to the identification of bacteria that can reduce disease severity. For example, *Pseudomonas* sp. WCS417 was identified in suppressive soils contributing biocontrol activity against the wheat fungal pathogen, *Gaeumannomyces graminis* var. *tritici* (30).

Understanding the specific genetic mechanisms used by beneficial microbes to protect their hosts against pathogens could help improve disease resistance and increase crop yields. Rhizosphere bacteria can use a variety of strategies to combat pathogens. These mechanisms include production of antibiotics, competitive exclusion, and manipulation of plant immune responses, which are each described below.

1.6.1 Mechanisms for bacterial-bacterial warfare (antibiosis)

Some PGPR can produce and secrete antimicrobial compounds conferring novel defense mechanisms against plant pathogens. Many *P. fluorescens* and *P. protegens* have been

characterized for their production of biocontrol compounds such as 2,4-diacetylphloroglucinol (DAPG), phenazine, and pyoluteorin (31). Genomic studies have identified a polyphyletic distribution of *Pseudomonas* strains that encode genes for 2,4-diacetylphloroglucinol (DAPG) (32, 33), an antifungal agent that can target a broad range of pathogens (15, 34). Many *Pseudomonas* spp., including plant-associated fluorescent Pseudomonads encode genes to produce bacteriocins, ribosomal polypeptides that can kill closely-related species (35, 36). Numerous *Pseudomonas* spp. isolated worldwide from rhizosphere soils or agricultural fields have also been found to produce phenazine derivatives, which have antimicrobial activity against a broad range of fungal and bacterial plant pathogens (33).

Many bacteria, including *Pseudomonas aeruginosa*, use quorum sensing to monitor their population levels and regulate gene expression through the production of mobile N-acylhomoserine lactone (AHL) signalling molecules. These include coordinating expression of genes involved in virulence or biofilm formation. Bacteria that can interrupt the quorum signalling of other species can gain a competitive advantage for their niche, and prevent their competitors from expressing virulence factors (37, 38).

1.6.2 Indirect protection through competitive exclusion and resource competition

Plants exude a variety of sugars, fatty acids, amino acids, secondary metabolites, and organic acids from their roots. The chemical composition of root exudates can influence rhizosphere microbiome assembly by selecting for bacteria that can metabolize the secreted compounds (39). Members of the rhizosphere microbiome can help reduce opportunities for pathogen infection through competitive exclusion, as pathogens must be able to outcompete existing microbiota. Competitive exclusion occurs when two bacteria compete for the same niche

and resources and one strain outcompetes the other (40). Therefore, the introduction of bacteria in the rhizosphere that can outcompete soil-borne pathogens for resources and space could be an effective biocontrol method (41, 42). Other genetic mechanisms relating to nutrient uptake or transport can influence bacterial fitness and survival in the rhizosphere. For example, bacteria that use additional or more efficient transporters to take up low-abundance nutrients may have a fitness advantage over competitors (43, 44).

Since iron is usually limited in soils, but an essential nutrient for bacterial growth, some beneficial microbes secrete siderophores that scavenge iron in the surrounding soil. These siderophores can either reduce or improve the iron available for other organisms, including soil-borne pathogens, depending on whether they have compatible transporters to take up the iron-bound siderophores (45, 46). These siderophores can play a role in resource competition between beneficial microbes, and lead to the suppression of pathogens.

1.6.3 Plant immune manipulation

Both beneficial microbes and pathogens can possess the same microbe-associated molecular patterns (MAMPs). However, upon detection of MAMPs belonging to pathogens, plants can often defend themselves through a pattern-triggered immunity (PTI) response (47). While pathogens such as *P. syringae* DC3000 evade PTI by secreting effectors using a type III secretion system (T3SS), some beneficial *Pseudomonas* strains also encode a similar T3SS (7, 48–50). It is still unclear how plants differentiate between MAMPs of beneficial and pathogenic bacteria, but it is likely that beneficial microbes are able to evade or suppress plant immune responses in order to colonize the roots (51–54).

The presence of certain bacteria in the rhizosphere can also trigger induced systemic resistance (ISR), which primes the plant immune system for future attack from pathogens or insect herbivores (55). How beneficial microbes trigger ISR is largely unknown; however, some studies have implicated the siderophore pyoverdine, bacterial lipopolysaccharides (LPS), volatile organic compounds, flagella, DAPG, and N-acyl homoserine lactones (AHL) (56).

Some beneficial bacteria in the rhizosphere can produce or manipulate phytohormone levels. Some can produce auxins such as IAA, which can stimulate lateral root and root hair formation to improve plant growth (57). However, bacterial auxin production can also dampen plant immune responses (58), which may improve root colonization by protective bacteria (59). Other bacteria can produce ACC deaminase, an enzyme which degrades the precursor to ethylene, resulting in an increase in root growth during stresses such as pathogen attack (60, 61). Plants face a fitness trade-off between growth and defence, so modulation of phytohormones by commensal microbiota may also impact the plant's ability to regulate this balance (54, 61, 62).

1.7 *Arabidopsis thaliana* and *Pseudomonas* as a model for plant-microbiome interactions

Host associations between the reference plant *Arabidopsis thaliana* and *Pseudomonas* species are commonly used as a model system to study plant-microbe interactions in the rhizosphere (63). *Pseudomonas fluorescens* and related species are widely plant-associated and are ubiquitously present in the rhizosphere microbiome. Both *A. thaliana* and *P. fluorescens* are non-obligate symbionts that can be readily cultured and studied in the lab. These organisms have sequenced genomes, with an abundance of genetic tools available, allowing for use of the *A. thaliana*-*P. fluorescens* model for mechanistic plant-microbe interactions research.

The inoculation of single strains of *P. fluorescens* on *A. thaliana* is sufficient to cause beneficial effects to the plant, such as promoting plant growth (64), inducing systemic resistance to pathogens or herbivores (65), or increasing resistance to salinity stress (66). However, accessions of *A. thaliana* can also vary in their ability to associate with different strains of *Pseudomonas*, which can provide them with different fitness advantages in the presence of pathogens (67). Therefore, inoculating individual strains of *Pseudomonas* onto *Arabidopsis* is a useful reductionist model for studying both bacterial and plant genes involved in beneficial plant-microbe interactions (68).

1.8 The genus *Pseudomonas* includes commensals and pathogens of plants and animals

Members of the genus *Pseudomonas* are Gram-negative γ-proteobacteria and possess the highest number of named bacterial species within a genus (69). The *Pseudomonas* genus is extremely diverse, and contains many host-associated bacteria that range from beneficial to pathogenic on plants and animals. Some examples include the opportunistic human pathogen *Pseudomonas aeruginosa*, the plant leaf pathogen *Pseudomonas syringae*, and ubiquitous soil and plant-associated bacteria *P. fluorescens*. *Pseudomonas* strains are also enriched in plant rhizospheres (70–72).

Although 16S rRNA gene sequencing is typically used to assign bacterial taxonomy, the 16S rRNA gene sequence lacks sufficient power to differentiate between strains within the *Pseudomonas* genus (73). Currently, the standard method of classifying phylogenetic relationships between *Pseudomonas* strains relies on multi-locus sequence analysis (MLSA), typically using sequences of housekeeping genes *gyrB*, *rpoB*, and *rpoD*, in addition to the 16S rRNA gene sequence (73). The genus *Pseudomonas* is divided into three main lineages, *P.*

fluorescens, P. aeruginosa, and *P. pertucinogena* (73). The *P. fluorescens* lineage is composed of five groups: *P. fluorescens, P. syringae, P. putida, P. asplennii,* and *P. lutea* (73). Even still, strains in the *P. fluorescens* group within the *P. fluorescens* lineage only share about 50% of their core genome, demonstrating a vast amount of genetic and functional diversity (7), and the *P. fluorescens* group can be further divided into eight sub-groups, including 75 named species and sub-species (73). The taxonomy within the *Pseudomonas* genus has a complex history, and genomic advances have caused additional reclassification and rearrangements of strains (69, 73).

1.8.1 Mining *Pseudomonas* genomes for plant-beneficial activity

Many plant-associated *P. fluorescens* have a variety of beneficial impacts on their hosts. As described previously, these include growth promotion, nutrient acquisition, and protection against pathogens, herbivores, and abiotic stresses (3, 15, 65, 74, 75). Computational genomic analyses can facilitate discovery of novel genes within plant-beneficial strains, generate hypotheses for genes required for plant-beneficial activity, and draw conclusions about the evolutionary history of these traits. A genomic analysis of 63 phenazine-producing *Pseudomonas* strains collected globally revealed the presence of many other genetic determinants for plant-beneficial activity, including DAPG, hydrogen cyanide (HCN), siderophore, and cyclic lipopeptide biosynthesis genes (33). A comparative genomics study of nine *Pseudomonas* strains with varying biocontrol ability against *Phytophthora infestans* identified genes whose presence correlated with anti-*Phytophthora* activity, including HCN, type II secretion, and a *higA/higB* toxin (76). Another comparative genomics analysis between ten *P. fluorescens* strains identified novel biocontrol genes previously not known to be present within those strains (36). Many of these biocontrol genes were found in regions with mobile genetic elements or repetitive

extragenic palindromic elements, possibly indicating that these loci were recently acquired (36). Collectively these studies indicate that phenotypic assays coupled with genomics can reveal novel traits in *Pseudomonas* that are important for plant health and pathogen protection.

1.8.2 *Pseudomonas* sp. FW300-N2C3 is an opportunistic pathogen within the Brassicacearum clade

The pathogen *Pseudomonas* sp. FW300-N2C3 (N2C3) is a member of the Brassicacearum clade of the *P. fluorescens* group. It causes detrimental effects on *A. thaliana* in axenic conditions (7). However, it is unable to cause disease in natural soil conditions (77). This suggests N2C3 is an opportunistic pathogen, only capable of infecting its host during advantageous circumstances, such as in the absence of a healthy rhizosphere microbiome (78).

N2C3 possesses a genomic island called the lipopeptide/quorum sensing (LPQ) island that is necessary for pathogenicity. A genome-wide association study (GWAS) conducted within the Brassicacearum, Corrugata, Mediterranea (BCM) clade of *P. fluorescens* revealed that the LPQ island is correlated with pathogenicity within this clade (Figure 1.4). This island contained genes encoding syringomycin and syringopeptin biosynthesis, as well as quorum signalling genes. This GWAS also uncovered two putative pathogenicity islets that were positively correlated with pathogenic lifestyle within the BCM clade. Conversely, three genomic regions were observed to be negatively correlated with pathogenicity. These included genomic regions encoding a type III secretion system (T3SS), the T3SS HopAA effector, and 2,4-diacetylphloroglucinol (DAPG) biosynthesis. These findings show that lifestyle transitions can occur over short evolutionary distances in *Pseudomonas* due to rapid gain and loss of genomic regions associated with beneficial or pathogenic traits.

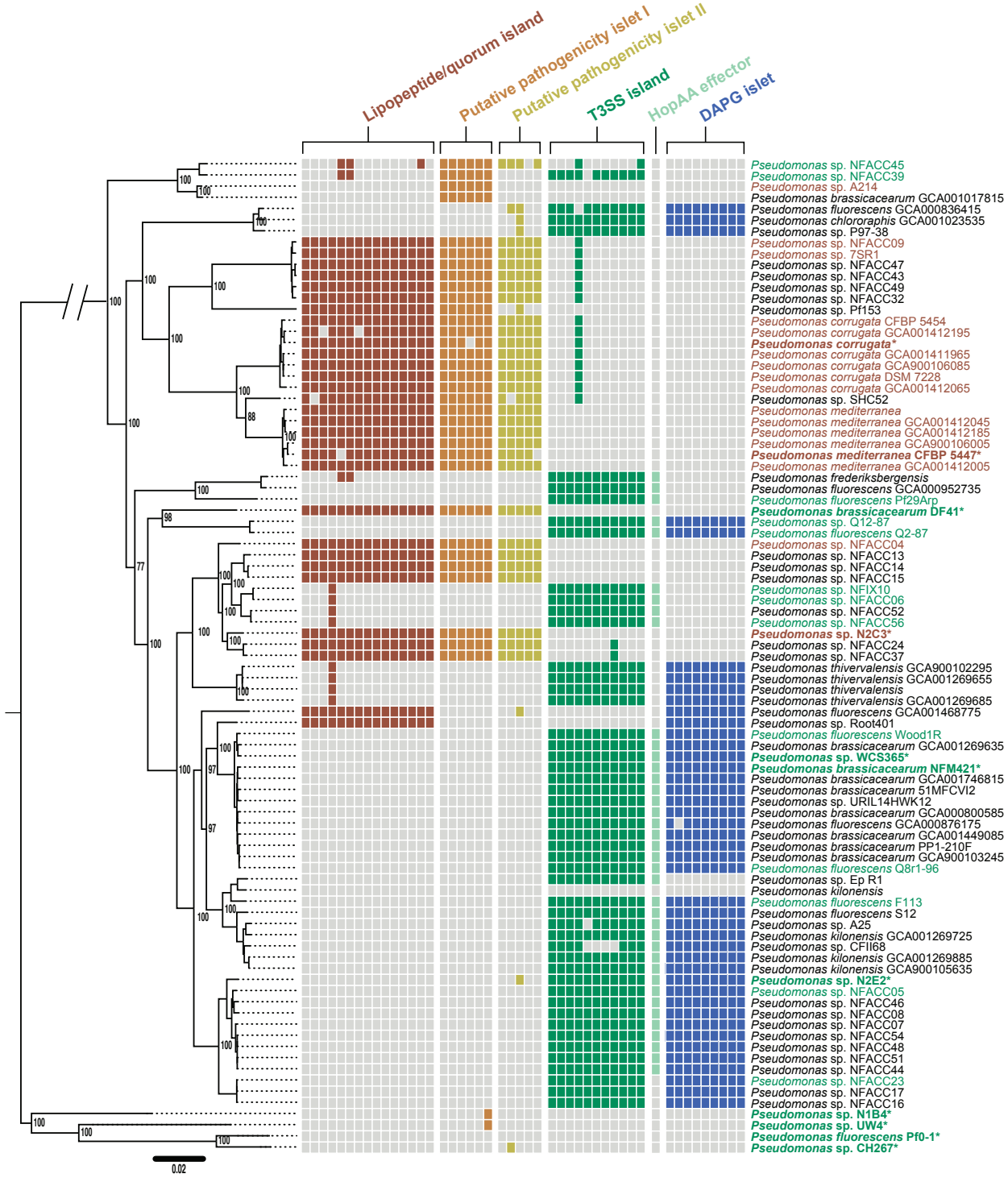

Figure 1.4 Polyphyletic distribution of pathogenic and commensal genomic islands within the BCM clade. Coloured squares represent the presence of individual genes associated with each genomic island in a strain. Strain names indicate pathogenic (red) or non-pathogenic (green) rhizosphere lifestyle based on experimental evidence (bold) or previous literature. In (© 2019 Springer Nature: The ISME Journal, reprinted and adapted with permission)

1.9 Objectives

While the pathogen *P. fluorescens* N2C3 readily causes disease under gnotobiotic conditions (7), it fails to cause disease in natural soil (77). I hypothesized that N2C3 was an opportunistic pathogen that could only cause disease in *Arabidopsis* in the absence of beneficial microbiota. In this study, I explored the potential of strains within the *Pseudomonas* genus to protect against this pathogen and identified the underlying genetic mechanisms necessary for protection.

1.9.1 Objective 1: Uncovering genomic regions associated with protection by *P. fluorescens*

My first objective was to screen phylogenetically diverse *Pseudomonas* strains for their ability to protect against N2C3, and uncover genomic regions associated with protection. I found that protection against N2C3 was largely limited to the *P. fluorescens* BCM clade. Since the members of the BCM clade are closely related, I hypothesized that they use the same mechanism to protect against N2C3. I also hypothesized that the genes required for protection against N2C3 are uniquely present in beneficial strains. To test these hypotheses, I categorized *Pseudomonas* strains based on their ability to protect against N2C3, then performed comparative genomics using PyParanoid, a pipeline developed in the Haney Lab (7) to identify genes associated with pathogen protection.

1.9.2 Objective 2: Identification of genes necessary for *Pseudomonas* sp. WCS365 to protect against *Pseudomonas* sp. N2C3

My second aim was to identify genes necessary for a model Brassicacearum strain, *Pseudomonas* sp. WCS365, to protect *Arabidopsis* from the pathogen N2C3. I used a reverse genetics approach to test whether known plant-beneficial traits are required for protection against N2C3. I tested genes involved in antimicrobial production, bacteria-plant signalling, and host colonization as well as candidate genes unique to protective *Pseudomonas* strains identified in Objective 1 (Chapter 1.9.1). Deletions were generated in the protective strain *Pseudomonas* sp. WCS365 and tested for their involvement in protection against N2C3.

Chapter 2: Rhizosphere *Pseudomonas fluorescens* protect *Arabidopsis* against an opportunistic pathogen through a colonization-dependent mechanism

2.1 Materials and Methods

2.1.1 Bacterial cultures and growth media

All *Pseudomonas* spp. were routinely cultured on King's B (KB) or lysogeny broth (LB) agar plates, and incubated at 28°C (Supplemental Table 1). *Escherichia coli* and *Chromobacterium violaceum* CV026 were cultured on LB agar plates, and incubated at 37°C and 28°C, respectively. Overnight cultures of *Pseudomonas* spp. and *C. violaceum* CV026 were prepared in 5 mL of LB, grown at 29°C, and shaken at 180 rpm. Overnight cultures of *E. coli* were prepared in 5 mL of LB, grown at 37°C, and shaken at 180 rpm. When required, growth media were supplemented with 25 μg/mL gentamycin, 10 μg/mL nalidixic acid, 10% sucrose, or 0.2 mg/mL 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal).

NFACC- and NFIX- strains used in this study were generously provided by Dr. Myoung-Hwan Chi and Dr. Kelly Craven of the Noble Foundation. They are a part of a bacterial collection isolated from the roots of Switchgrass (*Panicum virgatum*) growing in Tallgrass Prairie Preserve, Oklahoma, USA (79).

2.1.2 Plant growth conditions

Arabidopsis thaliana Col-0 seeds were sterilized by submersion in 70% ethanol for 2-3 minutes, then 10% bleach for 1-2 minutes. The seeds were rinsed three times using sterile deionized water, then suspended in 0.1% phytoagar. The seeds were stored in the dark at 4°C for

at least 2 days prior to sowing. The plants were grown in a growth room using a 16h light/8h dark cycle, under 100 μM fluorescent white light.

2.1.3 Axenic root inoculation assays

Sterilized seeds were planted onto square plates containing 0.5X Murashige and Skoog (MS) media, with 0.5 g/L 2-(N-morpholino)ethanesulfonic acid (MES) buffer, 2% sucrose, and 1% phytoagar. The plates were sealed using Micropore tape and placed upright, allowing seedlings to germinate along the surface of the media. After 6 days, seedlings were carefully transferred onto new square plates containing 0.5X MS media, with 0.5 g/L MES buffer, no sucrose, and 1% phytoagar, before being sealed and returned to the growth room. On day 7, seedlings were inoculated along their roots with 5 μL of bacterial treatments, prepared as described below. 5 seedlings were inoculated per treatment, and 2 treatments were tested per square plate.

Bacteria were prepared by inoculating single colonies into 5 mL of LB and grown overnight in a shaking incubator. 1 mL of overnight culture was centrifuged for 1 min at >10000 rcf, and the pelleted cells were resuspended in 1 mL of 10 mM $MgSO_4$. The resuspended cells were serially diluted to an estimated OD_{600} of 0.001 using 10 mM $MgSO_4$. Bacterial mixtures were usually prepared at 5:1 of test strain:N2C3. For example, a 5:1 treatment of WCS365:N2C3 would contain 50 μL of WCS365 and 10 μL of N2C3.

After bacterial inoculation, the plates of plants were resealed and the plants were grown vertically for 7 days in the growth room. Images of the plates were then scanned, and seedlings from the same treatment were pooled for fresh weight measurements (Supplemental Figure 1).

2.1.4 Rhizosphere CFU counts

N2C3-LacZ and WCS365-LacZ strains were generated through biparental mating of the N2C3 or WCS365 with *E. coli* WM3064 or CC18 containing *pMini-Tn5-lacZ* (79).

For quantification of rhizosphere colony-forming unit (CFU) counts, either N2C3 or WCS365 strains containing a *pMini-Tn5-lacZ* insertion were used to co-inoculate 7-day-old *Arabidopsis* seedlings, as described in the Axenic Root Inoculation Assay protocol (Chapter 2.1.3). After 7 days, seedlings were pooled and sterilely transferred into a 2 mL microcentrifuge tube containing a metal bead and 500 µL of 10 mM $MgSO_4$. Fresh weights were measured for each treatment. Plants were homogenized using a Qiagen TissueLyser II at 30 Hz for 2 min. Tissue lysate was serially diluted and plated onto LB agar supplemented with 0.2 mg/mL of X-gal. Plates were incubated at 28°C for 2 days. Blue and white CFUs were counted, to calculate the ratio of blue LacZ-containing cells and white co-inoculated cells in the rhizosphere (Supplemental Figure 2).

2.1.5 AHL biosensor assays

Overnight cultures of *Pseudomonas* sp. WCS365, *Pseudomonas* sp. N2C3, and *C. violaceum* CV026 were resuspended then diluted in 10 mM $MgSO_4$, to an OD_{600} of 2. Bacterial treatments were prepared by mixing 5:1, 1:1, and 1:5 ratios of WCS365:N2C3. Using an inoculation loop, bacterial treatments were streaked along an LB agar plate. CV026 was streaked beside each bacterial treatment. Plates were incubated at 28°C for 2 days. Plates were imaged and production of violacein by CV026 was recorded.

2.1.6 Comparative genomics using PyParanoid

Comparative genomics was performed using the PyParanoid pipeline described previously (7, 80). Briefly, the PyParanoid pipeline was previously used to create a database using 3886 *Pseudomonas* genomes, identifying 24066 homologous protein families ("gene groups") that covered 94.2% of the generated *Pseudomonas* pangenome. The presence or absence of gene groups were compared in strains with protection phenotypes determined via the Axenic Root Inoculation Assay (Chapter 2.1.3).

2.1.7 Phylogenetic trees

Phylogenetic trees were generated using the PyParanoid pipeline as described previously (7, 80). Briefly, an alignment of 122 single-copy genes conserved within the *Pseudomonas* genus was created previously. FastTree 2 was used to generate a phylogenetic tree from this alignment, by randomly sampling 5000 amino acid residues without replacement. Strains were then subset from this alignment to create phylogenetic trees, using FastTree 2.

2.1.8 Generating gene deletions

Gene deletions in WCS365 were created using a two-step allelic exchange method described previously (81). For each target gene deletion, 700-900 bp regions immediately flanking upstream and downstream of the target region were amplified using WCS365 genomic DNA as a template, and the primers listed in Supplemental Table 2. The amplified flanking regions were then joined using overlap PCR. The overlap PCR product was digested and ligated into the pEXG2 suicide vector, and transformed into chemically competent *Escherichia coli* DH5α cells. Correct insertions were confirmed by colony PCR and Sanger sequencing, before

transforming the plasmid into *E. coli* SM10λpir. Deletion plasmids were conjugated into WCS365 through biparental mating. Transconjugants that underwent a single crossover event allowing for site-specific chromosomal integration were selected for using LB agar supplemented with 25 μg/mL of gentamycin and 15 μg/mL of nalidixic acid. After restreaking and validating antibiotic resistance, transconjugants were grown overnight in no-salt LB at 37°C, and cells that underwent a double crossover to excise the plasmid backbone were selected by plating on LB agar supplemented with 10% sucrose. Successful gene deletions were confirmed using PCR, gel electrophoresis, and Sanger sequencing.

Gene deletions in N2C3 were created using a modified two-step allelic exchange protocol, which was described in detail previously (7).

2.1.9 Cross-streak assays

Bacteria were grown overnight in LB, at 28°C. Using a sterile inoculation loop, the first bacterial strain was streaked onto LB plates, directly from the overnight culture. The plate was allowed to dry, before streaking the second strain was streaked perpendicular to the first. The plate was incubated at 28°C, then imaged after 1 day.

2.1.10 Statistical analyses

All statistical analyses were performed using Prism 9 software. In general, competition assays were analyzed by one-way ANOVA and Tukey's HSD test and LacZ assays were analyzed using two-way ANOVA and Tukey's HSD test.

2.2 Results

2.2.1 Closely-related *Pseudomonas* strains protect from an opportunistic pathogen

It was previously observed that the *Pseudomonas* pathogen FW300-N2C3 (N2C3) readily causes disease under gnotobiotic conditions (7), but fails to cause disease in soil (77), suggesting that members of the microbiome may protect against pathogenesis. To test this, I used *Pseudomonas* sp. N2C3 and *Arabidopsis* as a model to identify bacterial strains that are protective against N2C3 pathogenesis in the rhizosphere under gnotobiotic conditions. Single strains of bacteria were co-inoculated with N2C3 onto gnotobiotic *Arabidopsis* seedlings to determine whether protection against N2C3 in natural soil could be attributed to the association with specific *Pseudomonas* strains in the rhizosphere microbiome. As the *Pseudomonas* genus is composed of genetically diverse species that are often present in the rhizosphere microbiome, 25 strains spanning the diversity of the *Pseudomonas* genus were tested for their ability to protect against N2C3 (Supplemental Table 1).

I identified 10 *Pseudomonas* strains that when co-inoculated at a 5:1 ratio with N2C3, increased the fresh weight of *Arabidopsis* (Figure 2.1). I also identified 11 strains that failed to protect against N2C3 as indicated by a similar fresh weight as seedlings treated with N2C3 alone. An additional 4 strains stunted plants, including two *P. aeruginosa* strains known to be pathogenic on plants (82, 83), and two *P. fluorescens*-clade stains that have a pathogenicity island encoding syringomycin and syringopeptin (7), indicating they are pathogenic. Collectively this indicates that commensal *Pseudomonas* can protect from an opportunistic pathogen under gnotobiotic conditions and that there is natural variation in the protective ability across the genus *Pseudomonas*.

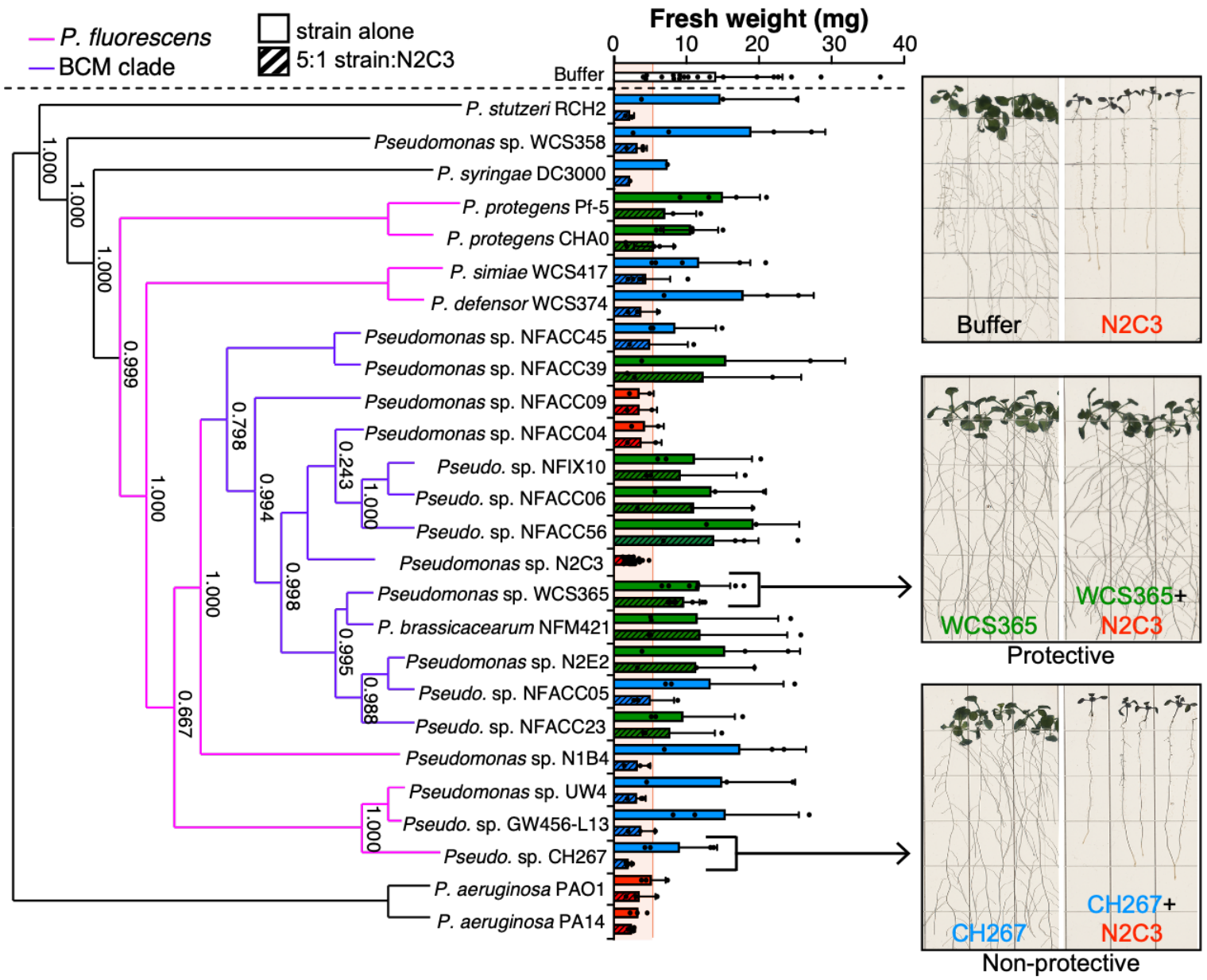
P. fluorescens
BCM clade
strain alone
5:1 strain:N2C3
Fresh weight (mg)
0
10
20
30
40
Buffer
P. stutzeri RCH2
Pseudomonas sp. WCS358
P. syringae DC3000
P. protegens Pf-5
P. protegens CHA0
P. simiae WCS417
P. defensor WCS374
Pseudomonas sp. NFACC45
Pseudomonas sp. NFACC39
Pseudomonas sp. NFACC09
Pseudomonas sp. NFACC04
Pseudo. sp. NFIX10
Pseudo. sp. NFACC06
Pseudo. sp. NFACC56
Pseudomonas sp. N2C3
Pseudomonas sp. WCS365
P. brassicacearum NFM421
Pseudomonas sp. N2E2
Pseudo. sp. NFACC05
Pseudo. sp. NFACC23
Pseudomonas sp. N1B4
Pseudomonas sp. UW4
Pseudo. sp. GW456-L13
Pseudo. sp. CH267
P. aeruginosa PAO1
P. aeruginosa PA14
1.000
1.000
1.000
0.999
1.000
0.798
0.994
0.243
1.000
0.998
1.000
0.667
0.995
0.988
1.000
Buffer
N2C3
WCS365
WCS365+
N2C3
Protective
CH267
CH267+
N2C3
Non-protective

Figure 2.1 Phylogenetically-related commensal *Pseudomonas* can protect against the *Pseudomonas* pathogen N2C3. The ability to protect against N2C3 (green) is prevalent within the Brassicacearum/Corrugata/Mediterranea (BCM) clade (purple). Beneficial *Pseudomonas* strains outside of the BCM clade, with the exception of Protegens strains Pf-5 and CHA0 are typically unable to protect (blue). *Pseudomonas* strains were co-inoculated with N2C3 at a 5:1 ratio. Fresh weight of *Arabidopsis* seedlings was used as a proxy for protection against N2C3. Each data point represents an average of 4-5 plants from a single experiment. Red bars indicate pathogenic strains that stunt plant growth when inoculated alone. Dashed line represents the mean + 3 standard deviations of N2C3-treated plants, estimating where 99.7% of measurements should fall under a normal distribution. Means that surpass this threshold were classified as protective (green bars). Representative images of buffer, pathogen (N2C3), protection (by WCS365) and lack of protection (by CH267) are shown.

I observed that 8 of the 10 protective *Pseudomonas* strains are within the Brassicacearum, Corrugata, and Mediterranea (BCM) clade (7) and the other two are within the Protegens clade (Figure 2.1) suggesting there may be some phylogenetic groups of *Pseudomonas* that are more able to protect against N2C3 pathogenesis than others. Using the data shown in Figure 2.1, I tested whether strains in the BCM and Protegens clade were significantly more likely to protect than other strains. I found a significant increase in *Arabidopsis* weights when seedlings were treated with strains from either the BCM or Protegens clades, but not other commensal *Pseudomonas* strains (Figure 2.2) supporting that strains within these phylogenetic groups are more likely to be protective against N2C3.

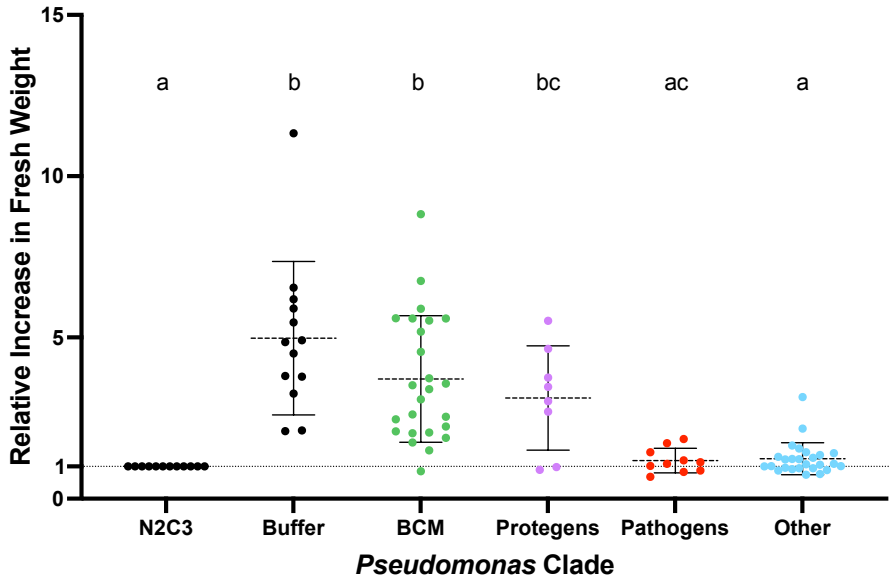

Figure 2.2 Strains within the BCM and Protegens clades significantly increase plant fresh weight, while other non-pathogenic strains do not. Each data point represents an average of 4-5 plants co-inoculated with the test *Pseudomonas* strain and N2C3 from a single experiment. Plant fresh weights were normalized by dividing by the average fresh weight of N2C3-treated control plants for the experiment. All repeated treatments tested in independent experiments were included as separate data points. Statistics were calculated using a one-way ANOVA and Tukey's HSD. Lines represent mean +/- SD.

2.2.2 Gain and loss of components of the *Pseudomonas* accessory genome does not explain protection against *Pseudomonas* sp. N2C3

A number of phenotypes within *Pseudomonas,* ranging from benefits to plants (7, 80), and virulence on plants and animals (7, 84), can be attributed to gain and loss of components of the *Pseudomonas* accessory genome. To identify genes that may correlate with protection, I used PyParanoid, a previously described comparative genomics platform (7), to identify genes whose presence or absence were indicative of protective or non-protective strains. However, I was unable to find novel genes through comparative genomics that explained protection, nor was I able to observe changes in protection due to genes known to be correlated to pathogenicity in BCM strains. Based on the results described in this section, I concluded that the gain or loss of

genes within the accessory genome of *Pseudomonas* does not explain the ability of certain strains to protect against N2C3.

2.2.2.1 Comparative genomics revealed that genes common to protective BCM strains and *P. protegens* Pf-5 were not necessary for protection against N2C3.

To determine if there are genes unique to protective BCM clade strains, the presence and absence of *Pseudomonas* gene groups was compared between seven protective BCM strains (NFM421, N2E2, WCS365, NFIX10, NFACC23, NFACC45, NFACC39), the protective non-BCM strain Pf-5, and a non-protective, beneficial strain CH267 using the PyParanoid pipeline. Both protective and non-protective BCM strains were included in this comparison, to prevent exclusion of genes that are differentially expressed within the BCM clade. 167 gene groups were common to these eight protective *Pseudomonas* strains, but absent in CH267. These genes were filtered further, eliminating 153 genes that were present in 6 additional *Pseudomonas* strains (WCS358, WCS417, WCS374, N1B4, GW456-L13, UW4) that had been tested to be non-protective. The remaining 14 genes (Figure 2.3, Table 2.1) are uniquely present in protective members of the rhizosphere microbiome, making them promising candidate genes to investigate the mechanisms involved in establishing protective microbiomes.

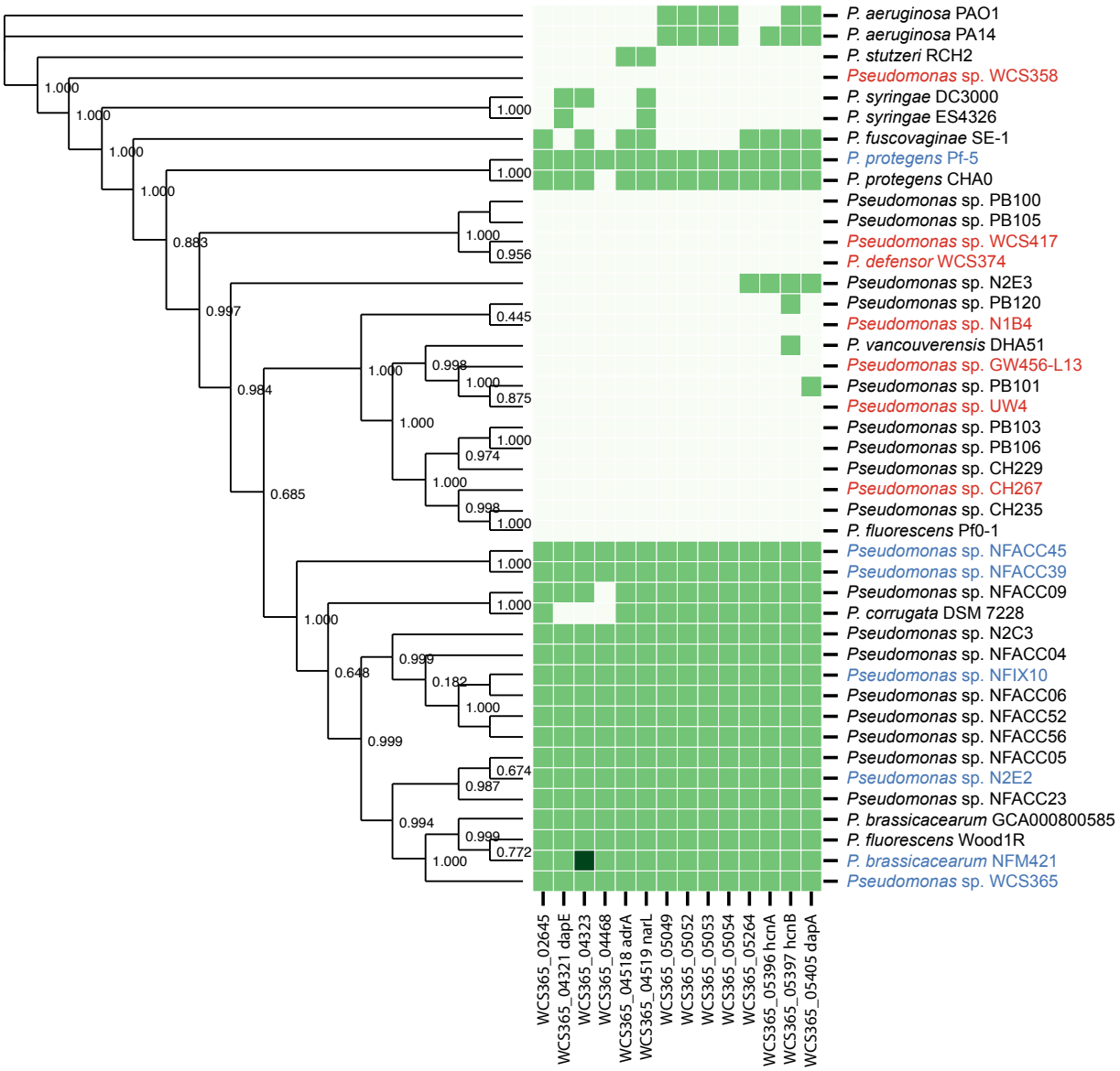

Figure 2.3 Comparative genomics identified 14 genes common to BCM strains and Pf-5. To test whether Pf-5 obtained genes responsible for protection in the BCM clade through horizontal gene transfer, I identified 14 genes that were present in 7 BCM strains and Pf-5 (blue labels), but absent in 7 non-protective strains outside of the BCM clade (red labels). Gene groups were labelled by their locus tag in WCS365.

Table 2.1 Comparative genomics revealed 14 genes unique to protective 8 *Pseudomonas* strains. These genes were identified as present in 7 BCM strains and *P. protegens* Pf-5, but absent in 7 non-protective strains. Locus tags indicate the genes in WCS365.

Locus Tag	Description	Gene Name	GO Terms	Deletion Name
WCS365_02645	Soluble epoxide hydrolase	-	Catalytic activity	-
WCS365_04321	Succinyl-diaminopimelate desuccinylase	*dapE*	Proteolysis, metallopeptidase activity, hydrolase activity	WCS365Δ*oppD-dapE*
WCS365_04323	Oligopeptide transport ATP-binding protein	*oppD*	Peptide transport, ATPase activity, nucleotide binding, ATP binding	
WCS365_04468	Putative metabolite transport protein	*nicT*	Transmembrane transport, integral component of plasma membrane	WCS365Δ*nicT*
WCS365_04518	Putative diguanylate cyclase	*ardA*	-	WCS365Δ*04518-9*
WCS365_04519	Putative transcriptional regulatory protein	*narL*	Phosphorelay signal transduction system	
WCS365_05049	(2Fe-2S)-binding protein	-	-	WCS365Δ*05049-54*
WCS365_05052	Response regulator	-	Catalytic activity	
WCS365_05053	Sensor domain-containing diguanylate cyclase	-	-	
WCS365_05054	ABC transporter ATP-binding protein	-	-	
WCS365_05264	ABC transporter substrate-binding protein	-	-	WCS365Δ*05264*
WCS365_05396	Hydrogen cyanide synthase subunit	*hcnA*	Iron-sulfur cluster binding, electron transfer activity	WCS365Δ*hcnAB*
WCS365_05397	Hydrogen cyanide synthase subunit	*hcnB*	Oxidation-reduction process, oxidoreductase activity	
WCS365_05405	4-hydroxy-tetrahydrodipicolinate synthase	*dapA*	Catalytic activity, lyase activity	-

To test if genes unique to protective BCM strains were required for protection, I deleted these genes in the protective strain *P. fluorescens* WCS365. Six deletion mutants, spanning 12 out of 14 of the unique genes identified through comparative genomics, were created and tested for their ability to protect against N2C3 (Figure 2.4; Supplemental Table 2). However, when co-inoculated with N2C3 at a 5:1 ratio, none of the WCS365 deletion mutants caused a significant reduction in *Arabidopsis* fresh weight and thus demonstrated similar levels of protection as wildtype WCS365. Furthermore, these deletion mutants did not have a significant difference in rhizosphere colonization or fitness in comparison to wildtype WCS365 (Supplemental Figure 3).

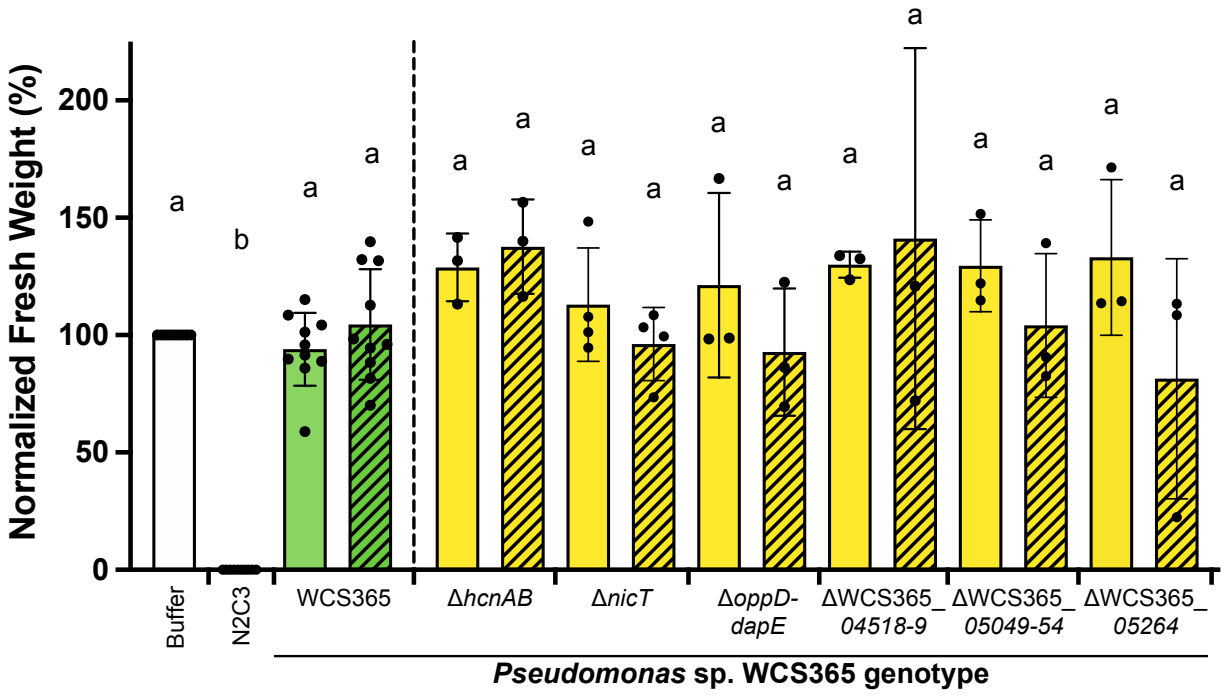

Figure 2.4 Candidate genes identified through comparative genomics between BCM strains and Pf-5 did not significantly impact protection against N2C3. 12/14 of the genes identified through comparative genomics described in Figure 2.3 were deleted in WCS365, but none of them had an impact on its ability to protect against N2C3. Data were normalized by dividing by average fresh weight of N2C3-inoculated plants. Statistics were calculated using a one-way ANOVA and Tukey's HSD. Mean +/- SD is plotted.

In order to exclude genes that may be present in non-protective BCM strains, I then searched for genes present in seven protective BCM strains (NFM421, N2E2, WCS365, NFIX10, NFACC06, NFACC39, and NFACC56), the protective non-BCM strain Pf-5, but absent in N2C3. This approach yielded only 1 unique gene (group_03914), encoding a CinA family protein (Supplemental Figure 4). However, this gene was subsequently found to be present in 9 non-protective strains (NFACC05, NFACC45, N1B4, PA14, PAO1, RCH2, WCS358, WCS374, and WCS417) and absent in the protective strain CHA0, indicating that it is unlikely to underlie protection. These comparisons suggest that Pf-5 did not gain its protective ability through horizontal transfer of genes from the BCM clade.

2.2.2.2 Other comparative genomics analyses did not reveal any promising candidate genes responsible for protection

I repeated the comparative genomics analyses using just protective and non-protective strains within the BCM clade, to identify if the presence of any gene groups were responsible for differences in protection ability within this clade. To identify genes unique to protective BCM strains, I searched for genes present in 8 protective BCM strains (NFM421, N2E2, WCS365, NFIX10, NFACC23, NFAC45, NFACC39, NFACC06, and NFACC56) but absent in 3 non-protective BCM strains (NFACC45, NFACC05, and N2C3). Using this approach, I identified no genes unique to the protective BCM strains (Supplemental Figure 5). To identify genes that are unique to non-protective BCM strains, I conducted the opposite analysis, searching for genes that were present in 3 non-protective BCM strains (NFACC45, NFACC05, and N2C3), but absent in 8 protective BCM strains (NFM421, N2E2, WCS365, NFIX10, NFACC23, NFAC45, NFACC39, NFACC06, and NFACC56). Using this approach, I identified 1 gene group encoding

an aldo/keto reductase (group_06773) that was unique to non-protective BCM strains (Supplemental Figure 6). However, when the presence/absence of this gene group was plotted onto a tree spanning the diversity of the *Pseudomonas* genus, I observed that not all non-protective strains outside of the BCM clade possessed this gene group, indicating that the absence of this gene was unlikely to be responsible for protection in the BCM clade.

To identify genes whose absence may be responsible for a BCM clade-specific mechanism of protection, I searched for genes that were present in 9 non-BCM strains (Pf-5, CH267, GW456-L13, N1B4, RCH2, UW4, WCS358, WCS374, and WCS417) but absent in 7 protective BCM strains (N2E2, NFACC06, NFACC39, NFACC56, NFIX10, NFM421, and WCS365) (Supplemental Figure 7). However, I was unable to identify any genes that met these criteria. These data suggested that genes uniquely present or absent in the protective or non-protective BCM clade members were not responsible for their ability to protect against N2C3.

2.2.2.3 Genes positively and negatively correlated with pathogenicity in the BCM clade are not necessary for protection

A previous GWAS identified three genomic regions that are negatively correlated with the LPQ pathogenicity island within the BCM clade, and are typically only present in the non-pathogenic members of the clade (Figure 2.1A) (7). These genomic regions encode a type III secretion system (T3SS), 2,4-diacetylphloroglucinol (DAPG) biosynthesis genes, and a T3SS effector, HopAA (7, 85). Although the T3SS is commonly used as a virulence mechanism in pathogenic *Pseudomonas* species, it has also been found in plant-beneficial strains, and has likely been gained in non-pathogenic BCM clade members through horizontal gene transfer events (7, 48).

To determine if the genomic regions unique to non-pathogenic BCM clade strains were necessary for protection against N2C3, I tested deletion mutants in WCS365, a beneficial Brassicacearum clade strain. The *hrcC* gene was deleted (79) to prevent the formation of the pilus required for secreting effectors via the T3SS (48). The DAPG biosynthesis island was also deleted in WCS365 (79). When each deletion mutant was co-inoculated onto *Arabidopsis* seedlings with N2C3, WCS365Δ*hrcC* and WCS365ΔDAPG maintained their ability to protect against N2C3 pathogenesis to a similar level as wildtype WCS365 (Figure 2.5B). This suggests that genes responsible for protection are not uniquely present in the non-pathogenic BCM strains.

To test whether the genomic regions correlated with pathogenicity in the BCM strains were responsible for a lack of protection, an avirulent N2C3 mutant that contained a deletion within the LPQ island, N2C3ΔSYRΔSYP, was also tested for its ability to protect against the wildtype N2C3. When co-inoculated with the wildtype pathogen, the avirulent N2C3ΔSYRΔSYP was unable to rescue seedling growth (Figure 2.5B). This reveals that protection in the BCM clade is not dependent on the absence of the LPQ pathogenicity island, or genes that are unique to pathogenic BCM strains.

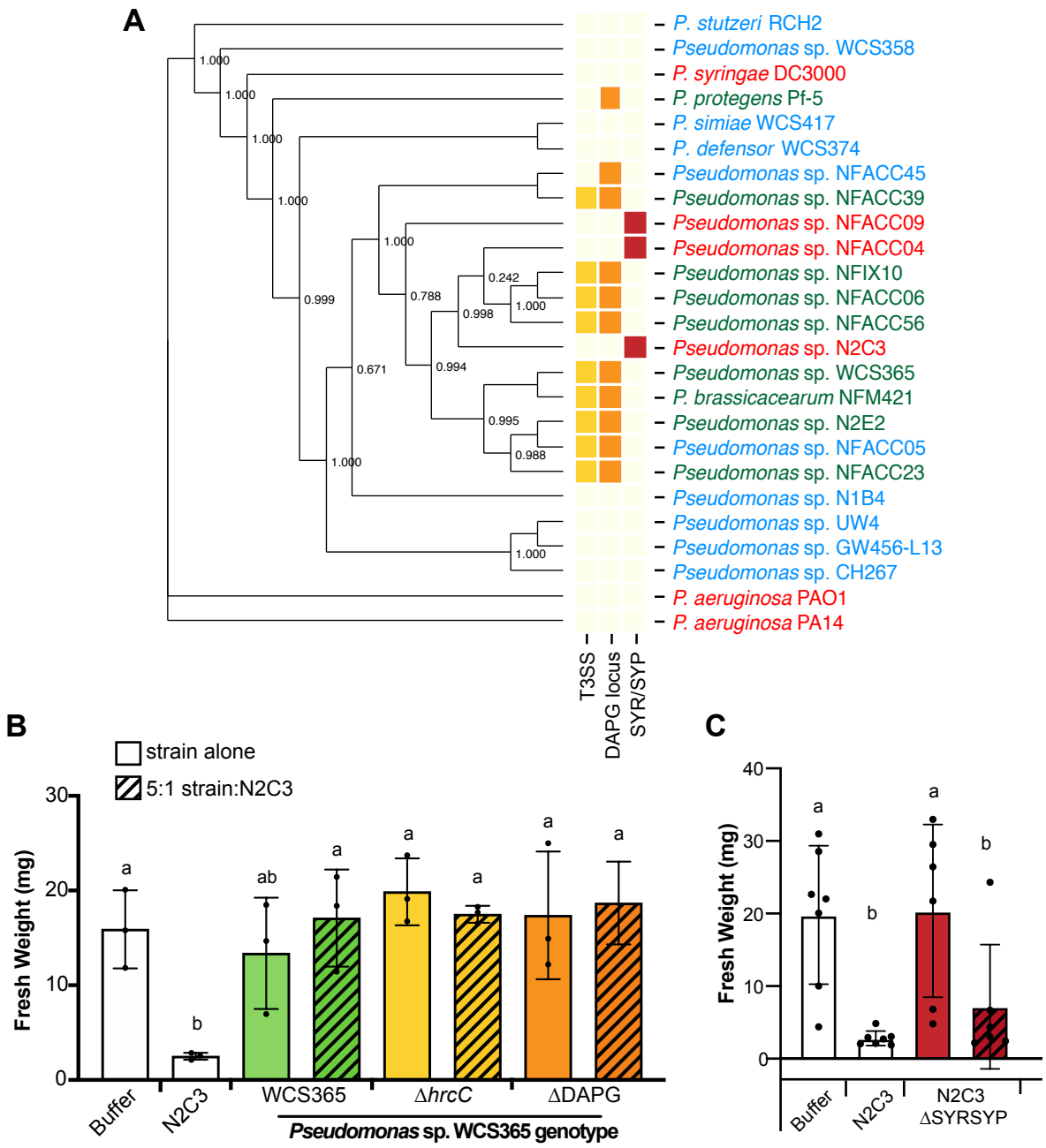

Figure 2.5 The ability to protect against pathogens is not due to horizontal transfer of components of the accessory genome. (A) A previous GWAS identified T3SS and DAPG biosynthesis genes that are anti-correlated with pathogenicity in the BCM clade (7). Strain names are colour-coded according to their ability to protect (green), not protect (blue) or stunt plants alone (red). (B) *Pseudomonas* sp. WCS365 Δ*hrcC* (deficient in T3SS) and ΔDAPG mutants still protect against N2C3. (C) Avirulent *Pseudomonas* sp. N2C3 cannot protect against virulent N2C3. A strain with a deletion of the pathogenicity island encoding syringomycin and syringopeptin was added at a 5:1 ratio with virulent N2C3. (B-C) Each dot represents the average weight of 4-5 plants from a single biological replicate. Letters indicate $p<0.05$ as determined using a one-way ANOVA and Tukey's HSD test. Lines represent mean +/- SD.

2.2.3 *Pseudomonas* sp. WCS365 does not inhibit N2C3 growth or quorum sensing *in vitro*

To gain insights into how protective strains may protect *Arabidopsis* from *Pseudomonas* sp. N2C3, I conducted *in vitro* experiments with the model protective BCM strain *Pseudomonas* sp. WCS365. While I had observed that WCS365 could protect *Arabidopsis* when co-inoculated with N2C3 in the rhizosphere, I wondered if WCS365 was directly killing or inhibiting the virulence of N2C3, and whether this could also occur outside of the rhizosphere. Using a bacterial cross streak assay, I found that WCS365 did not inhibit growth or kill N2C3 *in vitro* (Figure 2.6). This suggested that antagonism between WCS365 and N2C3 occurs only in the presence of the plant.

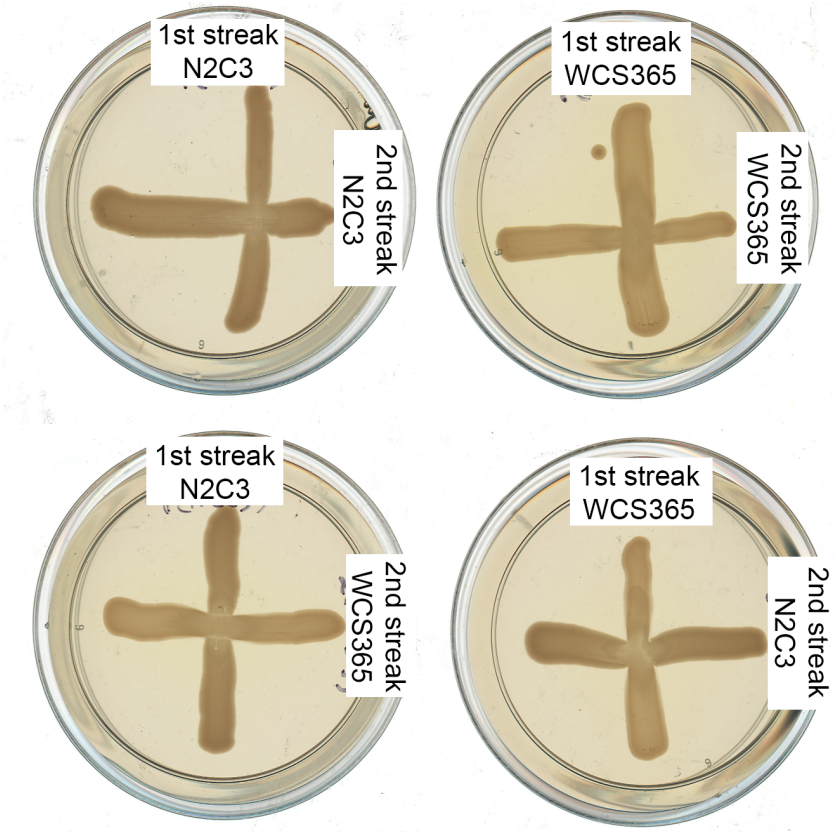

Figure 2.6 WCS365 and N2C3 do not inhibit each other's growth *in vitro*. Overnight cultures of WCS365 and N2C3, grown in LB, were streaked onto LB agar. The first strain was streaked vertically, and the second strain was streaked perpendicular to the first streak. Neither WCS365 or N2C3 created a zone of inhibition that prevented the growth of the strain that was streaked second.

While I did not find evidence that WCS365 could kill N2C3 *in vitro*, I wondered if it could interfere with its virulence. The LPQ island in N2C3 encodes the quorum signalling molecule, a short-chain N-acyl-homoserine lactones (AHL) synthase (*luxI*) and its corresponding AHL-binding transcriptional regulator (*luxR*) (7). These quorum signalling genes regulate syringomycin and syringopeptin production and are necessary for N2C3 pathogenicity (7). Meanwhile non-pathogenic, protective members of the BCM clade, such as the model strain *P. fluorescens* WCS365 do not contain the LPQ island, so they do not possess the quorum sensing genes present in N2C3. Since N2C3 pathogenicity is dependent on quorum sensing, I hypothesized that WCS365 and other protective BCM clade members could quench N2C3 quorum signals, therefore preventing biosynthesis of lipopeptides such as syringomycin and syringopeptin.

To test whether WCS365 can inhibit quorum signalling by N2C3, I used a *Chromobacterium violaceum* CV026 biosensor for C4-C8 AHL molecules, which reports AHLs from N2C3 through the production of the purple pigment violacein (Figure 2.7) (7, 86). Since WCS365 does not contain the AHL synthase genes present within the LPQ island, it does not trigger CV026 violacein production. I found that violacein production was maintained when WCS365 and N2C3 were mixed and plated onto LB agar, indicating that WCS365 does not suppress N2C3 quorum signalling (Figure 2.7). These data indicate that WCS365 does not directly kill N2C3 nor does it interfere with regulation of SYR/SYP-production in N2C3 *in vitro*.

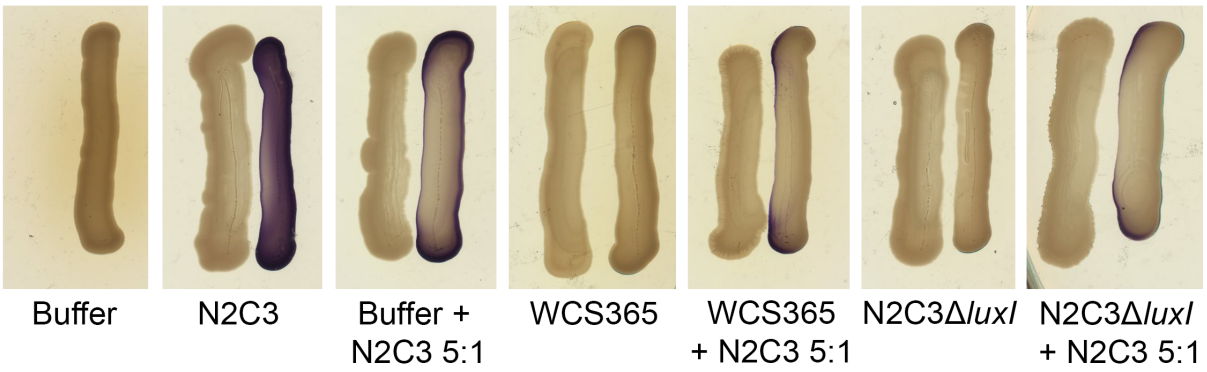

Figure 2.7 *Pseudomonas* sp. WCS365 does not inhibit quorum signalling of N2C3 *in vitro*. Although N2C3 virulence relies on quorum signalling genes (ex. the AHL synthase *luxI*) present in a pathogenicity island in the BCM clade (7), the protective WCS365 strain does not protect through quorum quenching. N2C3 AHL production is detected by the production of violacein, a visible purple pigment, by *C. violaceum* CV026. Each panel contains two streaks. On the left is the bacterial mixture described below each panel. On the right is the CV026 biosensor, which produces violacein in the presence of C4-C8 AHL molecules.

2.2.4 The ability of *Pseudomonas* strains to colonize correlates with their ability to protect against N2C3

Since I found that *Pseudomonas* sp. WCS365 does not kill N2C3 or inhibit quorum signalling *in vitro,* I hypothesized that protective strains may outcompete the pathogen in the rhizosphere. To test this, I used an N2C3 strain containing a LacZ transposon insertion (N2C3-LacZ) to allow me to homogenize roots and the attached bacteria, and distinguish CFUs from distinct *Pseudomonas* strains on media containing X-gal. I found that the protective strains (WCS365, N2E2 and Pf-5), maintained at least a 5:1 ratio (similar to the initial inoculum) in competition with N2C3-LacZ (Figure 2.8A). In contrast, CH267, which cannot protect *Arabidopsis* from N2C3, was significantly outcompeted by N2C3 (Figure 2.8A). WCS417, which also could not protect against N2C3, had a slight but not significant decrease in competition relative to the N2C3 control and the protective strains (Figure 2.8A).

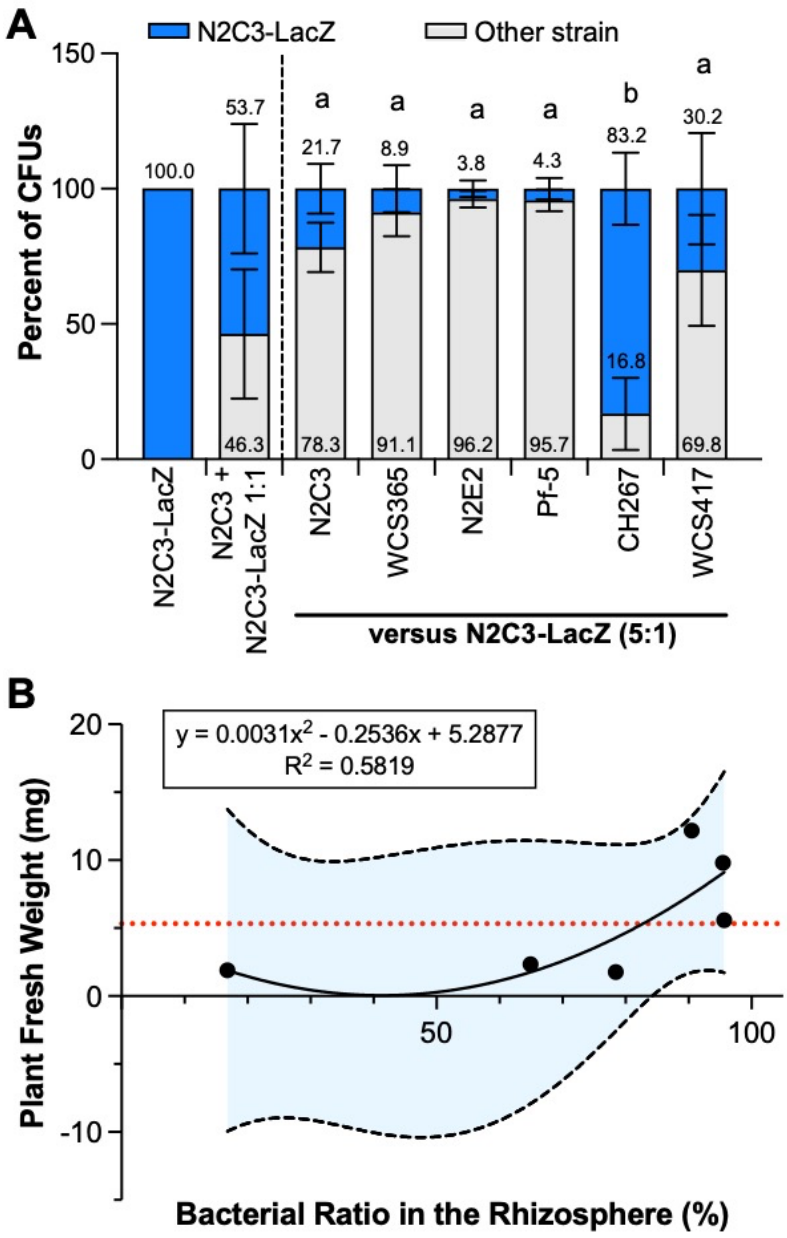

Figure 2.8 Protection against N2C3 is correlated with rhizosphere colonization. (A) Strains that protected against N2C3, such as members of the BCM clade (WCS365, N2E2) and Pf-5, maintained at least a 5:1 ratio (83%) of CFUs in competition with N2C3-LacZ. CH267, a strain that cannot protect, had decreased fitness when co-inoculated with N2C3-LacZ. Relative abundance of each strain was determined by grinding roots, plating on media containing X-gal, and counting blue and white colonies. Letters denote significance ($p<0.05$) by two-way ANOVA and Tukey HSD. Bars represent mean +/- SD. (B) Correlation between the bacterial fitness in the rhizosphere and the plant fresh weight. Data points represent the averaged fresh weights and average ratios for the strains co-inoculated with N2C3-LacZ described in (A). A polynomial linear regression was plotted with shaded area representing 95% confidence intervals. $R^2 = 0.5819$. The red dashed line denotes the upper 99.7% confidence interval for increase in plant weight when co-inoculated with N2C3 as determined in Figure 2.1.

I tested whether competition against N2C3 could predict protection in commensal *Pseudomonas* strains and found a polynomial linear relationship with a threshold for protection of approximately 5:1 (Figure 2.8B; Supplemental Figure 8). The model $y = 0.0031x^2 - 0.2536x + 5.2877$ explains about 58% of the plant fresh weight in response to bacterial colonization levels in the rhizosphere. These data indicate that colonization may be a prerequisite for pathogen protection among *Pseudomonas* spp. strains.

2.2.5 ColR-dependent colonization is necessary for protection against N2C3

Since I found that colonization was correlated with protection against *Pseudomonas* sp. N2C3, I hypothesized that competitive exclusion could be important for pathogen protection. During competitive exclusion, existing members of the microbiome protect against pathogens indirectly, by competing for space and nutrients. To test whether protection from N2C3 is colonization dependent, I tested 8 WCS365 deletion mutants that were found to have fitness defects in a previous Tn-seq screen (25) for their ability to protect against N2C3. I found that the majority of genes tested are broadly conserved across protective and non-protective strains (Supplemental Figure 9). Of the 8 mutants tested, I found that only WCS365Δ*colR* lost its ability to protect against N2C3 (Figure 2.9). Even when co-inoculated at a 50:1 ratio of WCS365Δ*colR*:N2C3, the Δ*colR* mutant was unable to protect, alluding to a *colR*-specific mechanism, rather than solely due to lower colonization levels (Supplemental Figure 10). ColR is a response regulator in a two-component system that has previously been shown to be necessary for colonization of plant roots (25, 87). This indicates that protection against N2C3 relies on a ColR-dependent mechanism for colonization.

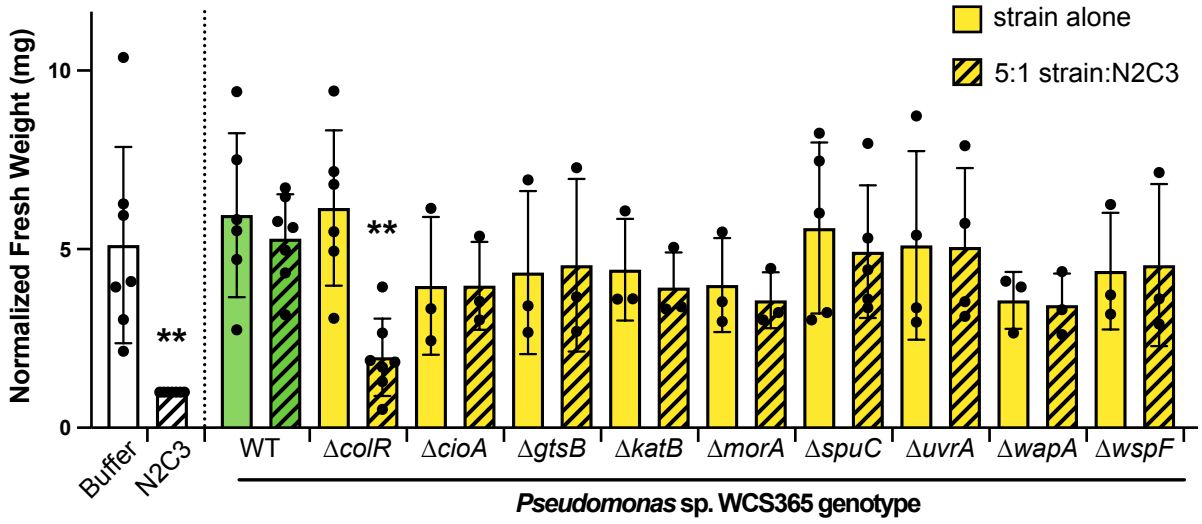

Figure 2.9 Deletion of the *colR* gene caused *Pseudomonas* sp. WCS365 to lose its ability to protect *Arabidopsis* from N2C3. WCS365Δ*colR* and seven additional WCS365 deletion mutants with colonization or rhizosphere fitness defects (25) were screened for their ability to protect *Arabidopsis* against N2C3. Each strain was co-inoculated onto roots with N2C3 at a 5:1 ratio. In comparison to plants co-inoculated with WCS365 and N2C3, plants co-inoculated with WCS365Δ*colR* and N2C3 were more stunted. Each strain was co-inoculated onto roots with N2C3 at a 5:1 ratio. **p<0.05 relative to WT WCS365 + N2C3 determined by one-way ANOVA and Tukey HSD. Mean +/- SD is plotted.

2.2.6 Two ColR-regulated genes are necessary for protection

Although ColR has previously been implicated in rhizosphere colonization, not much is known about the downstream processes it regulates. An RNAseq experiment identified genes that were putatively regulated by ColR (C.L. Wiesmann et al., unpublished data) (79). ColR is important for a variety of processes, including lipid homeostasis, membrane permeability, and cell signalling (23, 88). Six genes with ColR-dependent expression were deleted (Δ*cat*, Δ*eptA*, Δ*tpbA*, Δ*orf222*, Δ*wapQ*, Δ*02932*; C. L. Wiesmann, unpublished data) and were tested for their impact on protection against N2C3. Similar to *colR* and other colonization factors tested previously, these ColR-dependent genes are largely conserved within the *Pseudomonas* genus (Supplemental Figure 11). Amongst these genes, WCS365 deletions in Δ*wapQ* and Δ*02932*

caused a failure to protect against N2C3 (Figure 2.10). Although these genes are not well characterized, *wapQ* encodes a putative lipopolysaccharide kinase, and *WCS365_02932* encodes a putative phosphatidic acid phosphatase (23).

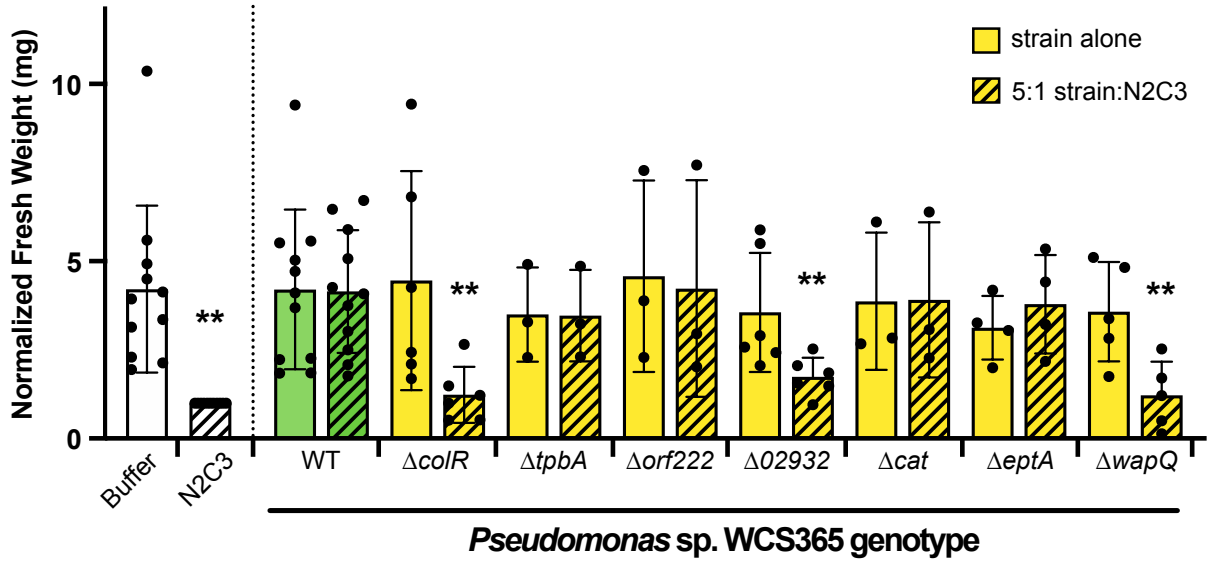

Figure 2.10 Protection against N2C3 is dependent on a subset of genes with ColR-dependent expression.
Two out of six ColR-dependent WCS365 deletion mutants were unable to protect against N2C3, when co-inoculated at a 5:1 ratio. Data were normalized by dividing by the average fresh weight of N2C3-inoculated control plants. **$p<0.05$ by one-way ANOVA and Dunnett's post-hoc test. Data for WCS365 mutants inoculated alone were excluded from the statistical analyses.

To determine if mutants that cannot protect against N2C3 also have competition defects with the pathogen, WCS365Δ*colR*, Δ*eptA*, Δ*wapQ*, and Δ*02932*, were tested for rhizosphere colonization levels when co-inoculated with N2C3 at a 5:1 ratio. Protective WCS365 and the WCS365Δ*eptA* mutant maintained their inoculated ratios in the rhizosphere, whereas the non-protective mutants WCS365Δ*colR*, WCS365Δ*wapQ*, WCS365Δ*02932* were significantly out-competed by N2C3 (Figure 2.11A). These data show that mutants that lose ability to compete against N2C3 also lose their ability to protect.

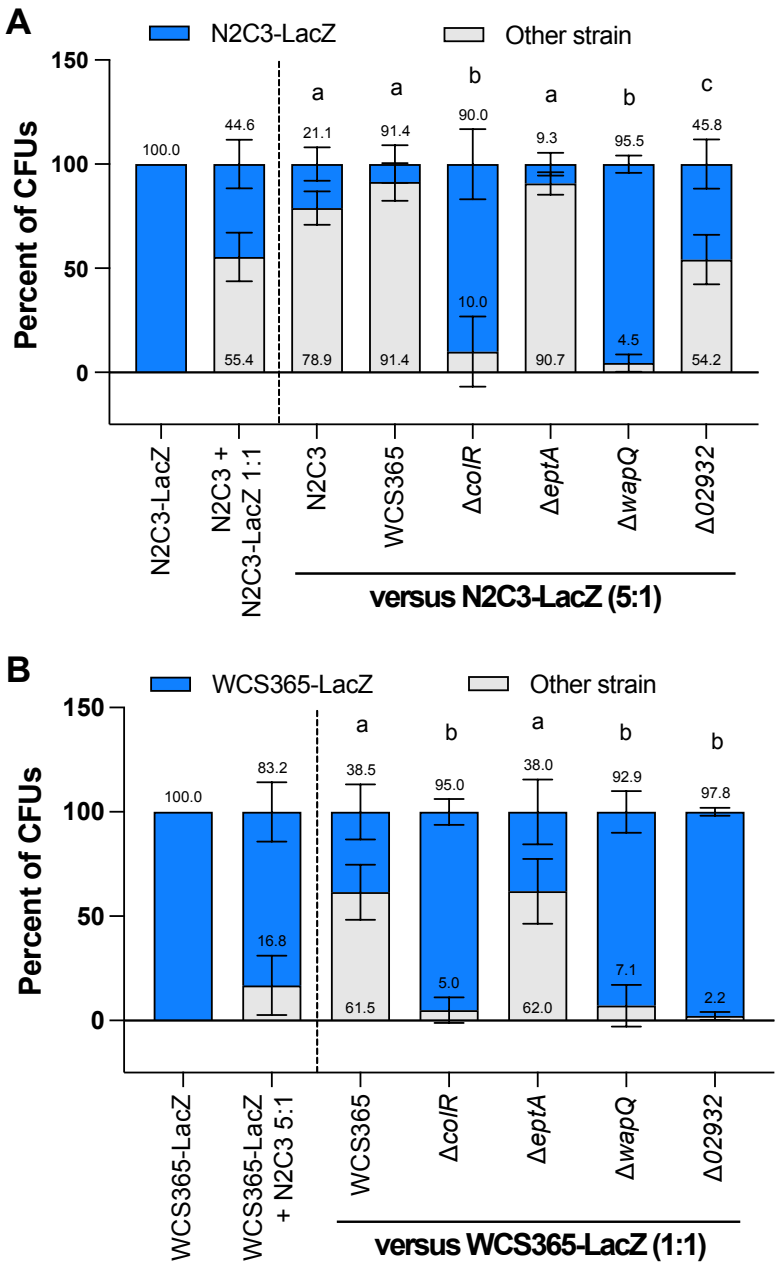

Figure 2.11 *Pseudomonas* sp. WCS365 mutants in non-protective ColR-dependent genes have decreased rhizosphere colonization and fitness. A) Loss of function mutants in genes with ColR-dependent expression that cannot protect against N2C3 at a 5:1 ratio (WCS365Δ*colR*, WCS365Δ*wapQ*, WCS365Δ*02932*) had decreased rhizosphere colonization and were outcompeted by N2C3. The WCS365Δ*eptA* mutant that can protect against N2C3, maintained approximately a 5:1 ratio (83%) of CFUs in the rhizosphere. B) Deletion of WCS365 genes that result in a protection defect against N2C3 also result in a competition defect in the rhizosphere. When competed against WCS365-LacZ at a 1:1 ratio, non-protective WCS365 deletion mutants (WCS365Δ*colR*, WCS365Δ*wapQ*, WCS365Δ*02932*) were significantly outcompeted. Protective mutants (WCS365Δ*eptA*) maintained a similar ratio in the rhizosphere compared to wildtype WCS365 when competed with WCS365-LacZ at a 1:1 ratio.

Mutants in ColR-regulated genes were also tested for rhizosphere fitness by competing them against a transgenic WCS365 containing a LacZ transposon insertion. When co-inoculated with WCS365-LacZ at a 1:1 ratio, 61.3% of the CFUs extracted from the rhizosphere were wildtype WCS365. The protective WCS365Δ*eptA* mutant maintained a similar percentage of CFUs in comparison to WCS365 (62.2%). Non-protective deletion mutants (WCS365Δ*colR*, WCS365Δ*wapQ*, and WCS365Δ*02932*) had a significant rhizosphere competition defect, making up less than 15% of CFUs (Figure 2.11B). These data support the observation that amongst diverse *Pseudomonas* strains and mutants, protection is correlated with rhizosphere colonization. Furthermore, only a subset of ColR-dependent genes, which have rhizosphere fitness defects, were found to be necessary for protection.

2.2.7 Commensal *P. fluorescens* strains can protect against other agricultural pathogens

To determine how broadly relevant the system I developed is for identifying members of the microbiome that can protect against *Pseudomonas* pathogens, I tested whether *Pseudomonas* sp. WCS365 was able to protect against other pathogens that use syringomycin and syringopeptin as virulence factors. I found that WCS365 resulted in a ~3-fold increase in the fresh weight of plants treated with *P. fuscovaginae* strain SE-1 (Figure 2.12A). Interestingly, although WCS365 increased fresh weight, shoot growth, and lateral root formation in the presence of SE-1, it did not fully rescue primary root stunting (Figure 2.12B). Collectively these data indicate that the described gnotobiotic assay may be able to broadly identify commensal strains and mechanisms that protect from agriculturally important pathogens.

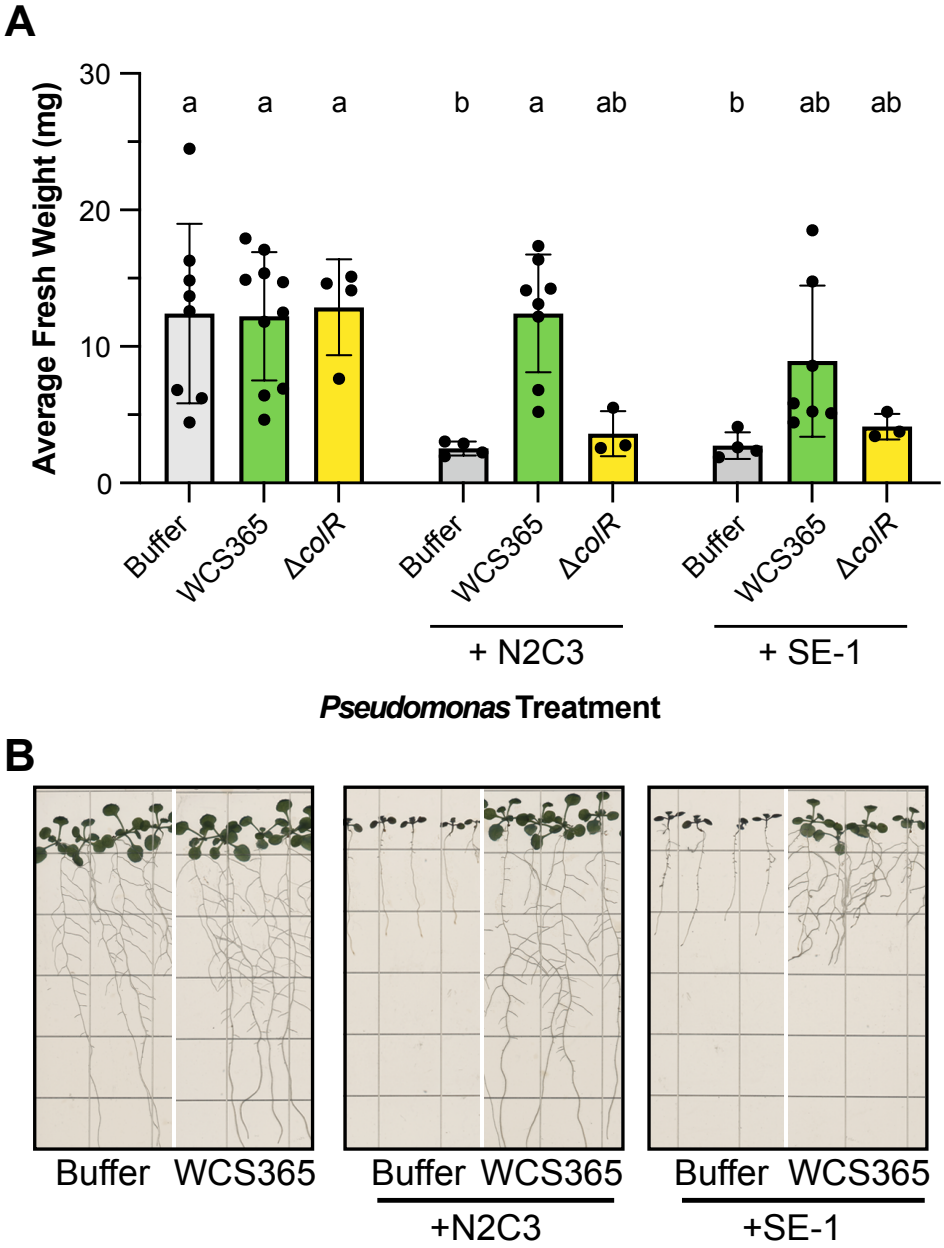

Figure 2.12 WCS365 protects against the agronomically important *Pseudomonas fuscovaginae SE-1* pathogen. (A) WCS365 protects against SE-1, the causal agent of bacterial sheath brown rot of rice when co-inoculated onto *Arabidopsis* roots at a 5:1 ratio. Letters indicate $p<0.05$ determined by one-way ANOVA and Tukey HSD. (B) Representative images of data quantified in (A).

WCS365 and other *P. fluorescens* strains were preliminarily screened for their ability to protect against other *Pseudomonas* pathogens that use syringomycin or syringopeptin (7, 89, 90).

WCS365 was tested for its ability to protect against other BCM clade pathogens, using a 2x increase in fresh weight when co-inoculated with the pathogen at a 5:1 ratio, as a threshold for protection. WCS365 could protect against *Pseudomonas* sp. NFACC09 and *P. mediterranea* CFBP 5447, but not *P. corrugata* DSM 7228 (Table 2.2). WCS365 was also able to protect against the agricultural pathogens, *P. fuscovaginae* strains SE-1, IRRI 7007, and IRRI 6609 (Table 2.2), which cause rice sheath brown rot (91).

Table 2.2 Preliminary screen for protection against other *Pseudomonas* pathogens. Beneficial *P. fluorescens* were tested for their ability to protect against *Pseudomonas* pathogens when co-inoculated onto *Arabidopsis* roots at a 5:1 ratio. Protection against N2C3 (Yes/No) was determined as described in Figure 2.1. Protection against other *Pseudomonas* pathogens was determined based on a 2x increase in fresh weight compared to plants inoculated with the pathogen alone. Experiments were conducted 1-3 times.

Pathogen	**Disease**	**Protection by co-inoculation with *Pseudomonas* strain (5:1)**				
		WCS365	NFM421	Pf-5	CH267	WCS365 Δ*colR*
Pseudomonas sp. N2C3	BCM clade pathogen	Yes	Yes	Yes	No	No
Pseudomonas sp. NFACC09	BCM clade pathogen	Yes	-	-	-	No
P. corrugata DSM 7228	Tomato pith necrosis; BCM clade pathogen	No	-	-	-	-
P. mediterranea CFBP 5447		Yes	-	-	-	No
P. fuscovaginae SE-1	Rice sheath brown rot	Yes	Yes	Yes	Yes	No
P. fuscovaginae IRRI 6609		Yes	-	-	-	-
P. fuscovaginae IRRI 7007		Yes	-	-	-	-

To determine whether a ColR-dependent mechanism was necessary for *Pseudomonas* protection against other pathogens, I tested the WCS365Δ*colR* mutant for its ability to protect against additional *Pseudomonas* pathogens. Using a 2x increase in fresh weight as the threshold for protection, WCS365Δ*colR* was unable to protect against any of the *Pseudomonas* pathogens I tested (Table 2.2; Figure 2.12). Based on this trend, these data suggest that beneficial members

of the rhizosphere microbiome may protect against a wider range of pathogens, through a ColR-dependent colonization mechanism. However, further replicates and statistical analysis should be performed to validate this finding.

Chapter 3: Discussion

3.1 There is a clade-specific mechanism within commensal *Pseudomonas* for protection against pathogenic *Pseudomonas* N2C3

Using a reductionist system consisting of the model plant *Arabidopsis,* an opportunistic *Pseudomonas* pathogen, and commensal *Pseudomonas* strains*,* I identified bacterial strains and genes required for members of the rhizosphere microbiome to protect against pathogens. Phylogenetically diverse strains across the *Pseudomonas* genus were screened for their ability to protect from *Pseudomonas* sp. N2C3 (Figure 2.1). I found that there was a phylogenetic signature to protection, and strains within the Brassicacearum, Corrugata, Mediterranea (BCM) clade were more likely to protect. Since strains within the BCM clade share a common ancestor, it is likely that they share genes that allow them to protect against N2C3. This supports the presence of a BCM clade-enriched mechanism that enables protection against N2C3 and potentially a broader range of opportunistic bacterial pathogens.

Since colonization was necessary for protection against N2C3, members of this clade likely have genes that improve bacterial colonization in the rhizosphere. These could include genes for improved nutrient uptake and metabolism, or for the evasion of plant defences, leading to competitive exclusion of the pathogen. Within the *Lactobacillus* genus, the presence/absence and expression levels of a manganese transporter gene *mntH1* has been correlated to the bioprotective ability against the dairy spoilage yeast *Debaryomyces hansenii* in fermented milk products (43). These bioprotective lactobacilli can competitively exclude *D. hansenii* in co-culture due to superior manganese scavenging and subsequently improved growth levels (43). This supports that competitive exclusion may be a common mechanism by which beneficial bacteria exclude pathogens.

3.2 The ability of *Pseudomonas* strains to protect against pathogenic N2C3 does not depend on genes unique to beneficial strains

Members of the BCM clade have genetic differences that cause differences in plant-associated lifestyles (7). The LPQ pathogenicity island encoding lipopeptide biosynthesis and quorum signalling genes is necessary for N2C3 pathogenicity, though it is still unknown whether these components are sufficient for pathogenicity (7). Since non-pathogenic BCM clade members do not possess the LPQ island-specific quorum signalling genes (7), it was possible that these strains protected by disrupting N2C3 quorum sensing. Quorum quenching has been observed as communication mechanisms amongst rhizobacteria, and a mechanism for biocontrol against plant pathogens (37, 92, 93). For example, *Pseudomonas segetis* P6 has been shown to degrade AHL signalling molecules of bacterial phytopathogens (94). Although I found that N2C3 quorum signalling was not affected by WCS365 *in vitro,* this does not rule out the possibility that beneficial strains might specifically inhibit quorum sensing in the rhizosphere.

Non-pathogenic members of the BCM clade contain genomic regions that are unique to non-pathogens suggesting that the ability to protect against N2C3 might have been due to the presence of these genes in protective strains. Deletion mutants in these genetic regions, WCS365Δ*hrcC* (defective in type III secretion) and WCS365ΔDAPG revealed that they were not necessary for protection against N2C3. These data provide supporting evidence that the genes involved in protection are not unique to the non-pathogenic BCM strains, but rather part of the *Pseudomonas* core genome.

3.3 Protection against pathogens may occur through differential expression of core *Pseudomonas* genes

Previous comparative genomics analyses have successfully identified phenotypes that show phylogenetic signatures in *Pseudomonas* and linked them to genomic islands in the accessory genome (7, 80, 84, 95). I performed several comparative genomics analyses between *Pseudomonas* strains using the PyParanoid pipeline, but was unable to find accessory genes whose presence or absence were necessary for protection against N2C3. For example, I tested a hypothesis that Pf-5 gained the ability to protect against N2C3 through horizontal gene transfer from the BCM clade, but found that deletions in genes identified through comparative genomics were not necessary for protection or rhizosphere fitness (Figure 2.4; Supplemental Figure 3), indicating that genes unique to BCM strains and Pf-5 are not required for protection.

Using the same method, comparative genomics via PyParanoid identified genomic islands necessary for pathogenicity and induced systemic susceptibility (7, 80). Melnyk et al. also subsequently performed a genome-wide association study (GWAS) on pathogenic and non-pathogenic BCM clade members reported in the literature, allowing for less stringent assessment of presence/absence data in order to infer correlations between genomic regions and pathogenicity (7). Ultimately, I was unable to identify components of the *Pseudomonas* accessory genome that correlate with protection. As a result, this work implicates variation in regulation of components of the core genome in protection against an opportunistic pathogen.

While comparative genomics is a powerful tool for identifying genetic mechanisms that are the result of the presence or absence of a gene or genomic region (80, 96), it is unable to detect expression-level differences or allelic variations. Allelic variations and differential gene expression can have significant effects on bacterial phenotype. Point mutations and differential

gene expression, which would not have been discernable by these analyses, can cause dramatic differences in *Pseudomonas* phenotype. For instance, increased expression levels of two genes involved in rRNA modification and stress response improved rhizosphere fitness of *P. putida* K2440 (97). A point mutation in the *gacA* two-component response regulator of *P. fluorescens* PfA-QS161 is also able to convert the strain from a non-edible commensal strain into a food source for the amoeba *Dictyostelium discoideum* (98). Phase variation, including spontaneous mutations in the *gacA*/*gacS* two-component system, also plays a role in rhizosphere colonization and biocontrol abilities in *Pseudomonas* (99). For example, two phases have been observed in *P. brassicacearum* NFM421 that impact rhizosphere colonization and have differences in motility, auxin production, and root localization (100). As I found that components of the *Pseudomonas* core genome contribute to protection against N2C3, this suggests that differential regulation of these genes may underlie the BCM clade-specific protection against N2C3.

While I found that *colR*-dependent colonization was necessary for protection against N2C3 and *Pseudomonas fuscovaginae* SE-1 by the protective BCM strain WCS365, *colR* is widely conserved within the *Pseudomonas* genus, and is present in both pathogenic and non-pathogenic *Pseudomonas* strains. Therefore, the presence of *colR* is not a clade-specific gene involved in protection, but could be the core genome component required for protection. Since Pf-5 is able to outcompete N2C3 in the rhizosphere (Figure 2.8A), similar to members of the BCM clade, protection against N2C3 by Pf-5 might be colonization and ColR-dependent. There may be a mechanism of regulating *colR* or *colR*-dependent gene expression that is common to protective members of the BCM and Protegens clades.

Although the BCM clade-specific mechanism was not identified through comparative genomic analyses performed in this research project, multiple avenues have been explored and

eliminated, and future screening methodologies may build on these results to reveal novel insights into this mechanism.

3.4 Pf-5 may use a different a genetic mechanism from the BCM clade to protect against N2C3

Although Pf-5 is able to outcompete N2C3 in the rhizosphere (Figure 2.8A), it has not been tested directly for *colR*-dependent protection against N2C3. While the ability to outcompete N2C3 could suggest that Pf-5 has superior colonization ability, it does not eliminate the possibility that Pf-5 suppresses N2C3 through antibiosis or triggering plant-immunity. Therefore, it is possible that Pf-5 uses an independent mechanism to protect against N2C3, rather than a *colR*-regulated mechanism like the BCM clade.

P. protegens Pf-5 has been reported to produce an abundance of biocontrol agents and secondary metabolites, including pyrrolnitrin, pyoleuterin, 2,4-diacetylphloroglucinol (DAPG), hydrogen cyanide (HCN), toxoflavin, rhizoxin analogues, pyochelin, and pyoverdine (101–103). It was first isolated and described due to its ability to suppress *Rhizoctonia solani* through the production of the antibiotic pyrrolnitrin (104). While members of the Brassicacearum clade have been reported to encode genes for DAPG, HCN, pyoverdine, similar to Pf-5, not all production of secondary metabolites overlap between the two clades (36, 105). Therefore, it is possible that Pf-5 uses an independent mechanism to protect against N2C3, rather than a *colR*-regulated mechanism like WCS365.

3.5 Protection against N2C3 requires interactions with the plant host

In cross-streak assays performed *in vitro* on LB, WCS365 was unable to prevent N2C3 growth (Figure 2.6). However, I found that WCS365 outcompeted N2C3 in experiments performed in the rhizosphere, and that rhizosphere colonization was correlated with protection in the *Pseudomonas* genus (Figure 2.8). While cross-streak assays cannot quantitively ascertain growth rates over time, it is possible that LB media, in which all my *in vitro* experiments were performed, is rich in nutrients that are limited in the rhizosphere, allowing both N2C3 and WCS365 to grow effectively *in vitro*. For example, though strains of lactobacilli could competitively exclude the spoilage yeast *Debaryomyces hansenii* under normal milk fermentation conditions, addition of excess manganese was able to restore *D. hansenii* growth (43). Similarly, WCS365 may be able to utilize a rhizosphere nutrient that N2C3 does not use as efficiently, and competitive exclusion is being masked under nutrient-rich *in vitro* conditions. These data collectively indicate that competition by BCM clade strains occurs most robustly in the presence of the plant.

While commensal bacteria can restrict pathogens *in vitro*, efficacy is often reduced when tested *in planta* (106). Therefore, it is reasonable to speculate that the opposite effect could occur. Genes involved in protection might be induced by the presence of plant-derived exudates or signals that would not be present *in vitro*. This is further supported by my discovery that *colR* is necessary for protection (Figure 2.9). As ColR is the response regulator of a two-component system, the associated sensor ColS could induce ColR phosphorylation and DNA binding in response to these plant-derived signals. While the plant-derived signal sensed by ColS is still unknown, ColS has been shown to respond to metal ions such as Fe^{3+} and Zn^{2+} in *P. putida* and *P. aeruginosa* to increase tolerance to metal stresses (88, 107). As a result, it is possible that

ColR-dependent antagonism against N2C3 is specifically triggered in the presence of a plant host.

3.6 Root colonization is a prerequisite for protection against N2C3

I found that rhizosphere colonization was correlated with protection (Figure 2.8), and antagonism was only observed in the presence of the plant, indicating that protective strains might only inhibit N2C3 growth after colonizing plant roots. Since the majority of protective strains were closely-related to N2C3, it is reasonable to suspect that protection occurs via competitive exclusion, as closely-related strains might overlap the most with N2C3 in resource usage.

Resource overlap has been shown to play a role in invasiveness of pathogens in a community (108). Furthermore, endophytic fungi have been proposed as biocontrol against the elm tree pathogen *Ophiostoma novo-ulmi* due to their significant nutritional niche overlap (42). N2C3 pathogenicity is dependent on syringomycin biosynthesis (7), which is regulated by iron and inorganic phosphate availability (109). Since these nutrients are often limited in the environment, members of the BCM clade may protect against N2C3 through competition for iron or phosphate. For example, by restricting iron from pathogen *Ralstonia solanacearum*, microbial consortia of siderophore-producers can reduce disease incidence in tomato plants (46). Therefore, competitive exclusion by closely-related bacteria, especially through competition for limited nutrients such as iron or phosphate, might explain protection against N2C3.

While beneficial *Pseudomonas* may protect against N2C3 through competitive exclusion, host colonization could also just be a prerequisite before releasing antibiotics or influencing plant defence responses. Antibiotic production in commensal rhizobacteria can be impacted by root

exudate composition and quorum signal accumulation (110), implying induction of antibiotic biosynthesis genes after rhizosphere colonization. For example, root exudates from the plant host *Eruca sativa* induce expression of the antibiotic bacillaene by *Bacillus subtilis*, allowing for killing and out-colonization of *Serratia plymuthica*, and improvement of systemic resistance to the pathogen *P. syringae* DC3000 (111). Commensal bacteria colonizing the gut and airway microbiomes reduce infection by stimulating components of the human innate and adaptive immune system (112, 113). As plants and animals share some similarities in their innate immune responses (114), and commensal bacteria vary in their ability to modulate plant innate immune responses (53, 54), it is possible that certain commensal bacteria could play a role in stimulating plant innate immunity upon encountering a pathogen.

3.7 ColR-regulated colonization is necessary for protection against N2C3

I found that rhizosphere colonization was correlated to protection against N2C3 (Figure 2.8), and that the colonization factor *colR* was necessary for protection (Figure 2.9). This alludes to WCS365 and other members of the BCM protecting by competitive exclusion. However, it was surprising to find that not all colonization factors or ColR-regulated genes were necessary for WCS365 to protect against N2C3 (Figure 2.9; Figure 2.10), indicating that a colonization mechanism due to ColR-dependent gene expression is required for protection against N2C3. This may mean that changes in ColR-dependent gene expression could have higher activity or greater impact on colonization in protective BCM clade members, whereas other *Pseudomonas* strains could employ redundant colonization factors.

In this study, *colR*, *wapQ*, and WCS365_*02932* were demonstrated to be necessary for protection by WCS365. *colR* was previously reported to be necessary for rhizosphere

colonization by affecting membrane permeability via *wapQ* (23). Meanwhile, *WCS365_02932* encodes a putative phosphatidic acid phosphatase, which has been shown to play a role in lipid synthesis in *E. coli* (115). Lipid homeostasis impacts the stability and efficacy of membrane transport proteins (44). Therefore, deletions in *colR*, *wapQ*, and *WCS365_02932* may impact nutrient uptake through the cell membrane, and subsequently reduce fitness in the rhizosphere.

3.8 N2C3 is an opportunistic pathogen that persists commensally in the rhizosphere

Protective strains co-inoculated with N2C3 tend to maintain approximately the same co-inoculation ratio, 7 days after inoculation (Figure 2.8A). This demonstrates that N2C3 is not entirely killed off by the protective strains, but rather persists in the rhizosphere as a commensal member of the microbiome. This could suggest that N2C3 swaps between pathogenic and non-pathogenic lifestyles, which is characteristic of opportunistic pathogens (78). This also suggests that a lack of soil diversity could lead to increased opportunities for pathogens to attack (116). Increased species richness (number of different taxa in a community) and functional dissimilarity (usage of different niches) within *P. fluorescens* communities have been shown to decrease the ability of the community to be invaded by *P. putida* IsoF (117). Non-virulent *Ralstonia* spp. communities with low nestedness (resource overlap between generalists and specialists), high connectance (the proportion of consumer-resource links that occur, amongst all possible consumer-resource links; a measure of links between species and resources available), and niche overlap with the pathogen could reduce infection by *Ralstonia solanacearum* in the tomato plant rhizosphere (108). Therefore, the presence of a diverse rhizosphere microbiome could increase species richness, functional dissimilarity, and the likelihood of niche overlap with pathogens, thus posing a larger challenge to pathogen infection.

3.9 Protection through competition/competitive exclusion may protect against a wide range of pathogens.

Closely-related *Pseudomonas* strains with markers of both commensal and pathogenic lifestyles have been identified within the microbiome of the same plant (7, 70, 79), suggesting competition may be ecologically relevant. I found that rhizosphere colonization via *colR* was necessary for WCS365 to compete against N2C3, and may also confer protection against SE-1 and related *P. fuscovaginae* strains. This indicates that the protective mechanism discovered in this study could be applied to combat a wider range of pathogens. This system may be broadly relevant for identifying strains and mechanisms to control agriculturally important pathogens.

3.10 Future directions

Since a BCM clade-specific mechanism for protection was not identified through the presence or absence of genes, RNAseq or qRT-PCR could be performed to determine if there is differential gene expression amongst protective and non-protective BCM strains. This may reveal clade-specific expression of genes, or differential regulation of *colR*-dependent genes.

Functional characterization of *colS, wapQ* and WCS365*_02932* should be performed in order to elucidate the effects of lipid homeostasis on colonization and protection. Analysis of root exudate profiles might reveal the plant-derived signals sensed by ColS needed to trigger ColR phosphorylation. Additional ColR-dependent genes could also be investigated for their involvement in protection against N2C3, to help narrow down the downstream pathways responsible. Deletion mutants should be made in other protective BCM or Protegens strains to

confirm whether *colR* is necessary for other *Pseudomonas* strains to protect *Arabidopsis* from N2C3.

Although I suspect that beneficial bacteria are protecting against opportunistic pathogens through competitive exclusion, further research should also be conducted on the rhizosphere-specific signals that lead to *in vivo* protection. This could include testing plant gene expression or exudate profiles in response to pathogen infection, or identification of the rhizosphere signal sensed by the ColS sensor.

It would also be interesting to investigate the Protegens clade further to identify its mechanism for protection against N2C3. Additional Protegens strains should also be tested for their ability to protect against N2C3. This could reveal whether protection is unique to Pf-5 and CHA0, or more abundant within the Protegens clade. RNAseq analysis could also be used to test differences in gene expression during protection by Pf-5 versus protection by the BCM clade.

In order to assess the broad-scale applications of protective BCM clade members, future work should also be conducted on studying whether *colR* and ColR-dependent genes such as *wapQ* and WCS365*_02932* are important for protection against a wider range of agricultural pathogens, including *P. fuscovaginae* SE-1. Additional *Pseudomonas* strains should also be screened, to better correlate whether protection against N2C3 is a good predictor of protection against these agricultural pathogens. It would be useful to explore how protection ability would change if the pathogen and the beneficial *Pseudomonas* were inoculated at different times. Lastly, inoculation of protective bacteria in synthetic communities could also be explored, especially in soil settings.

3.11 Conclusions

By identifying the bacterial genes required to colonize specific plants, and the plant genes required to shape their associated communities, microbiome engineering strategies could be applied during plant breeding. Conventional breeding practices rely solely on variation in the plant genome to produce cultivars with desirable traits when challenged with pathogens or abiotic stresses. Over time, crops have been bred to grow in dysbiotic soil systems with lowered microbial diversity. This could lead to plants losing the ability to attract key members of the microbiome that could provide benefits in response to environmental stressors. However, incorporating the plant microbiome into breeding strategies introduces additional genetic variation that could be used to select for improvements in agronomically important traits. When breeding plants, it would be useful to propagate plants alongside key members of their microbiome, to help them maintain desirable plant-microbe interactions once introduced to their new environment (Figure 3.1). This starter microbiome could consist of a single PGPR inoculant, an artificial community of PGPR, or microorganisms introduced from a soil sample (118). However, it is difficult to design synthetic, interconnected communities consisting of desirable PGPR strains without in-depth knowledge of the genetic mechanisms involved.

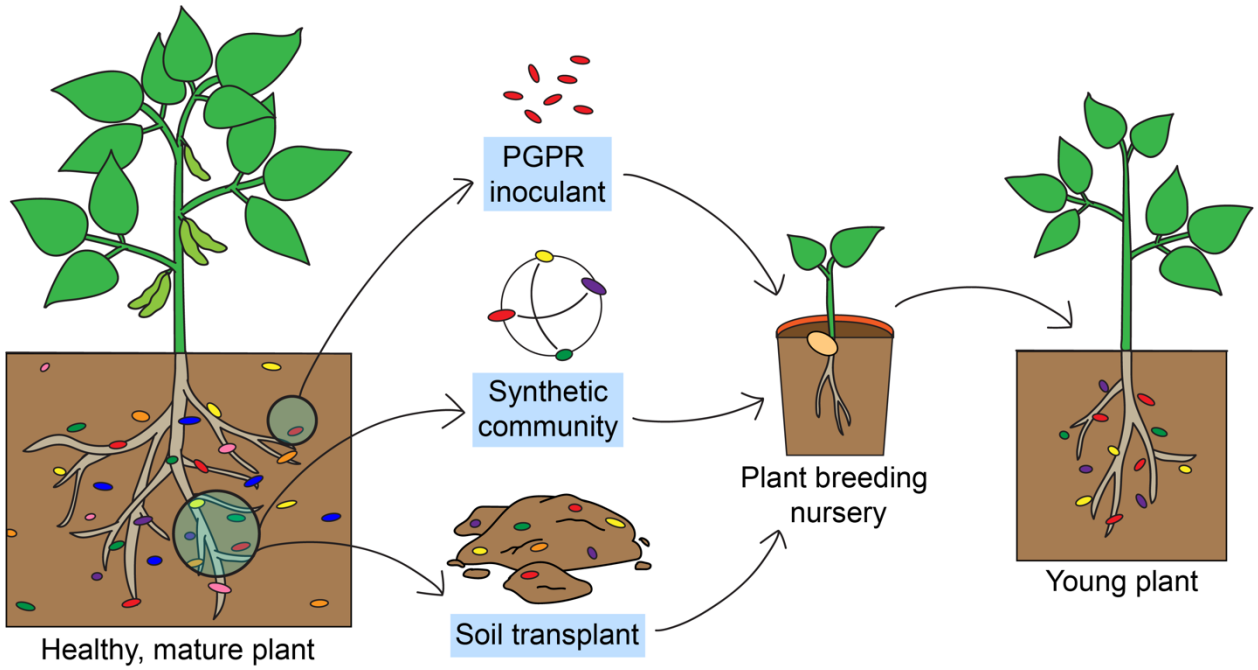

Figure 3.1 Overview of ways to incorporate microbiome engineering into agricultural cultivation practices. Microorganisms can be incorporated into the plant microbiome during the breeding stage, prior to large-scale cultivation. These microorganisms can be introduced by transplanting soils directly from the rhizosphere of healthy plants. Alternatively, the beneficial microorganisms in these soils can be analyzed to identify PGPR. These PGPR can be formulated as a single PGPR inoculant, or as a synthetic community of PGPR, and introduced to plants during breeding.

Ideally, the microbiome could be modified to provide protection against a broad range of conditions, such as multiple abiotic stresses, pathogens, and insect herbivores, in addition to maintaining or improving growth. However, all of these processes are intertwined, and are often controlled by modulating levels of the same few hormones. This means that increased resistance in one area can come with trade-offs, such as increased sensitivity to another factor. For example, increased resistance to insects due to jasmonic acid production can come at a cost to salicylic acid-mediated defence against bacterial pathogens (65). Therefore, it would be more feasible to develop microbial consortia to target the conditions of specific fields, on a case-by-case basis, rather than applying a generic consortium.

Future areas of research should include improving stable colonization of PGPR, untangling complex interactions between key microbes in a system, and incorporating the plant microbiome into breeding strategies. Microbiome engineering could play a vital role in harnessing the genetic potential of the plant microbiome and prevent future crop loss due to abiotic stresses and pathogen infection.

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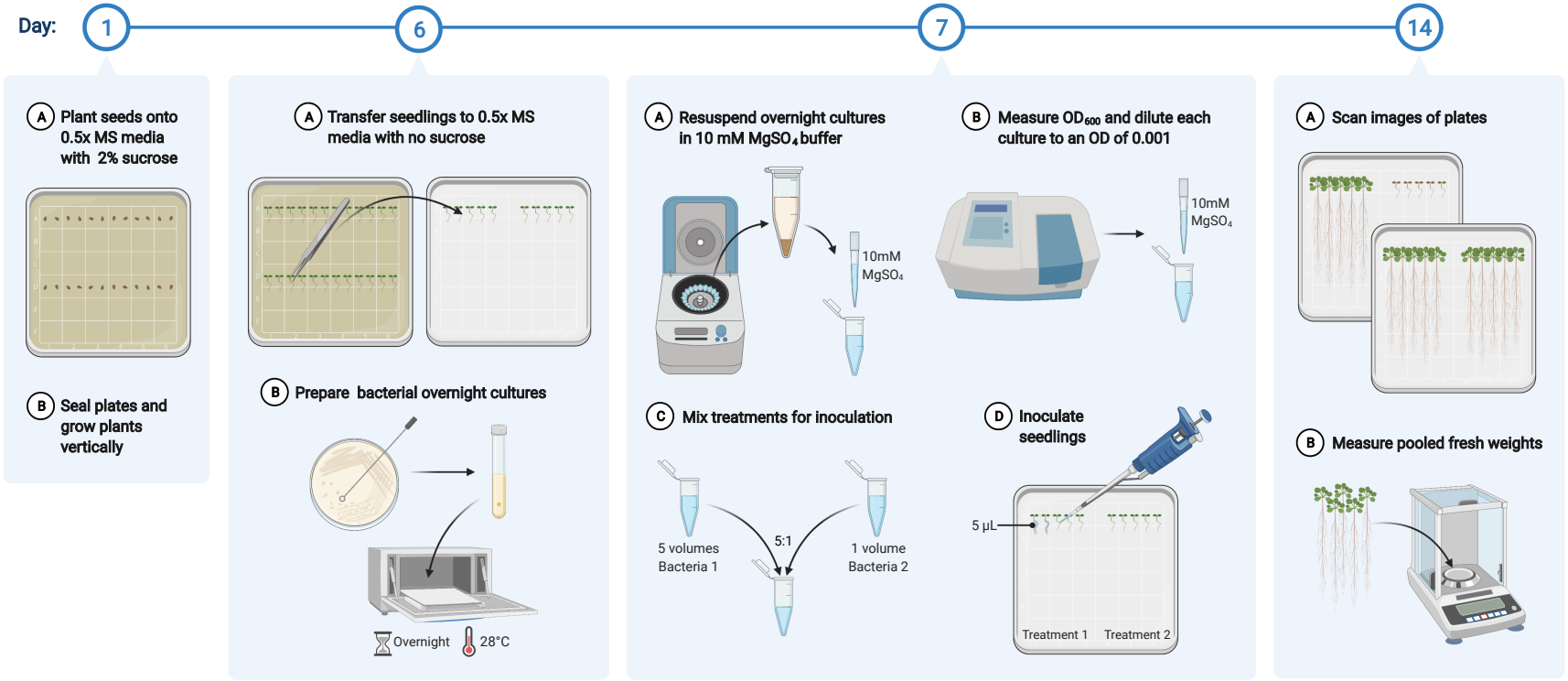
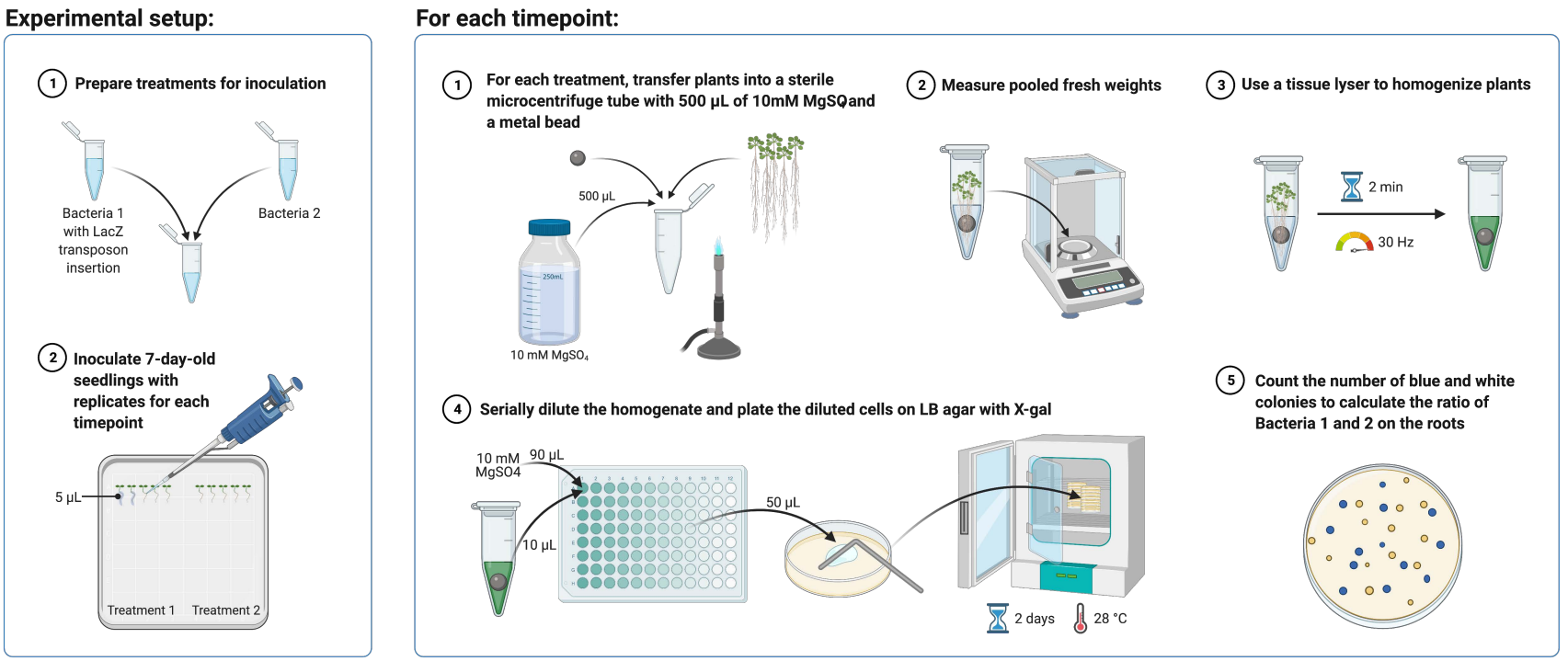
Appendix – Supplementary Figures and Tables

Supplemental Table 1 *Pseudomonas* strains used in this study.

Strain Name	Source	Location	Reference
P. mediterranea CFBP 5447	Tomato, pith necrosis	Italy	(119)
P. protegens CHA0	Tobacco rhizosphere	Switzerland	(120)
Pseudomonas sp. CH267	*Arabidopsis* rhizosphere	Cambridge, MA, USA	(67)
P. syringae DC3000	Tomato leaves	Guernsey, UK	(121, 122)(121, 122)
P. corrugata DSM 7228 (also referred to as CFBP 2431)	Tomato, pith necrosis	England	(123)
Pseudomonas sp. FW300-N1B4	Groundwater	Oak Ridge, TN, USA	(124)
Pseudomonas sp. FW300-N2C3	Groundwater	Oak Ridge, TN, USA	(124)
Pseudomonas sp. FW300-N2E2	Groundwater	Oak Ridge, TN, USA	(125)
Pseudomonas sp. GW456-L13	Groundwater	Oak Ridge, TN, USA	(124)
P. fuscovaginae IRRI 6609	Rice seed, sheath brown rot	Davao City, Philippines	(126)
P. fuscovaginae IRRI 7007	Rice seed, sheath brown rot	Aborlan, Philippines	(126)
Pseudomonas sp. NFACC04	Switchgrass rhizosphere	Osage County, OK, USA	(79)
Pseudomonas sp. NFACC05	Switchgrass rhizosphere	Osage County, OK, USA	(79)
Pseudomonas sp. NFACC06	Switchgrass rhizosphere	Osage County, OK, USA	(79)
Pseudomonas sp. NFACC09	Switchgrass rhizosphere	Osage County, OK, USA	(79)
Pseudomonas sp. NFACC23	Switchgrass rhizosphere	Osage County, OK, USA	(79)
Pseudomonas sp. NFACC39	Switchgrass rhizosphere	Osage County, OK, USA	(79)
Pseudomonas sp. NFACC45	Switchgrass rhizosphere	Osage County, OK, USA	(79)
Pseudomonas sp. NFACC56	Switchgrass rhizosphere	Osage County, OK, USA	(79)
Pseudomonas sp. NFIX10	Switchgrass rhizosphere	Osage County, OK, USA	(79)
P. brassicacearum NFM421	*Arabidopsis* rhizoplane	Méréville, France	(127)
P. aeruginosa PA14	Human wound	Boston, MA, USA	(128)
P. aeruginosa PAO1	Human wound	Melbourne, Australia	(129)
P. protegens Pf-5 (previously *P. fluorescens* Pf-5)	Cotton rhizosphere	College Station, TX, USA	(104)
P. stutzeri RCH2	Groundwater	Hanford, WA, USA	(130)
P. fuscovaginae SE-1	Rice seed, sheath brown rot	Siniloan, Philippines	(126)
Pseudomonas sp. UW4	Reed rhizosphere	Waterloo, ON, Canada	(131)
Pseudomonas sp. WCS358	Potato rhizosphere	Netherlands	(132, 133)
Pseudomonas sp. WCS365	Potato rhizosphere	Netherlands	(132)
P. defensor WCS374	Potato rhizosphere	Netherlands	(132, 133)
P. simiae WCS417	Wheat rhizosphere	Netherlands	(134)



Supplemental Figure 1 Axenic root inoculation protocol. On Day 1, sterilized seeds were planted in 2 rows onto 0.5x MS media with 0.5 g/L MES, 2% sucrose, and 1% phytoagar. The plates were sealed with Micropore tape and grown upright. On Day 6, seedlings were transferred onto 0.5x MS media with 0.5 g/L MES, no sucrose, and 1% phytoagar. 10 seedlings were transferred per plate, leaving a gap between each set of 5 plants. The plates were sealed and grown vertically. Bacteria were inoculated into LB and incubated overnight at 28°C, 180rpm. On Day 7, bacteria were centrifuged and resuspended in 10mM $MgSO_4$. After measuring the OD_{600}, the bacteria were serially diluted in 10mM $MgSO_4$ to an estimated OD of 0.001. Bacterial mixtures were prepared using ratios of a test strain:pathogen. For example, a 5:1 treatment of WCS365:N2C3 would contain 50 µL of WCS365 and 10 µL of N2C3. For each treatment, 5 plants were inoculated with 5 µL of bacterial treatment along the length of their roots. After inoculation, plates were resealed and grown vertically for 7 days. On Day 14, images of the plates were scanned, and plants from each treatment were pooled and weighed. Figure created with Biorender.com.

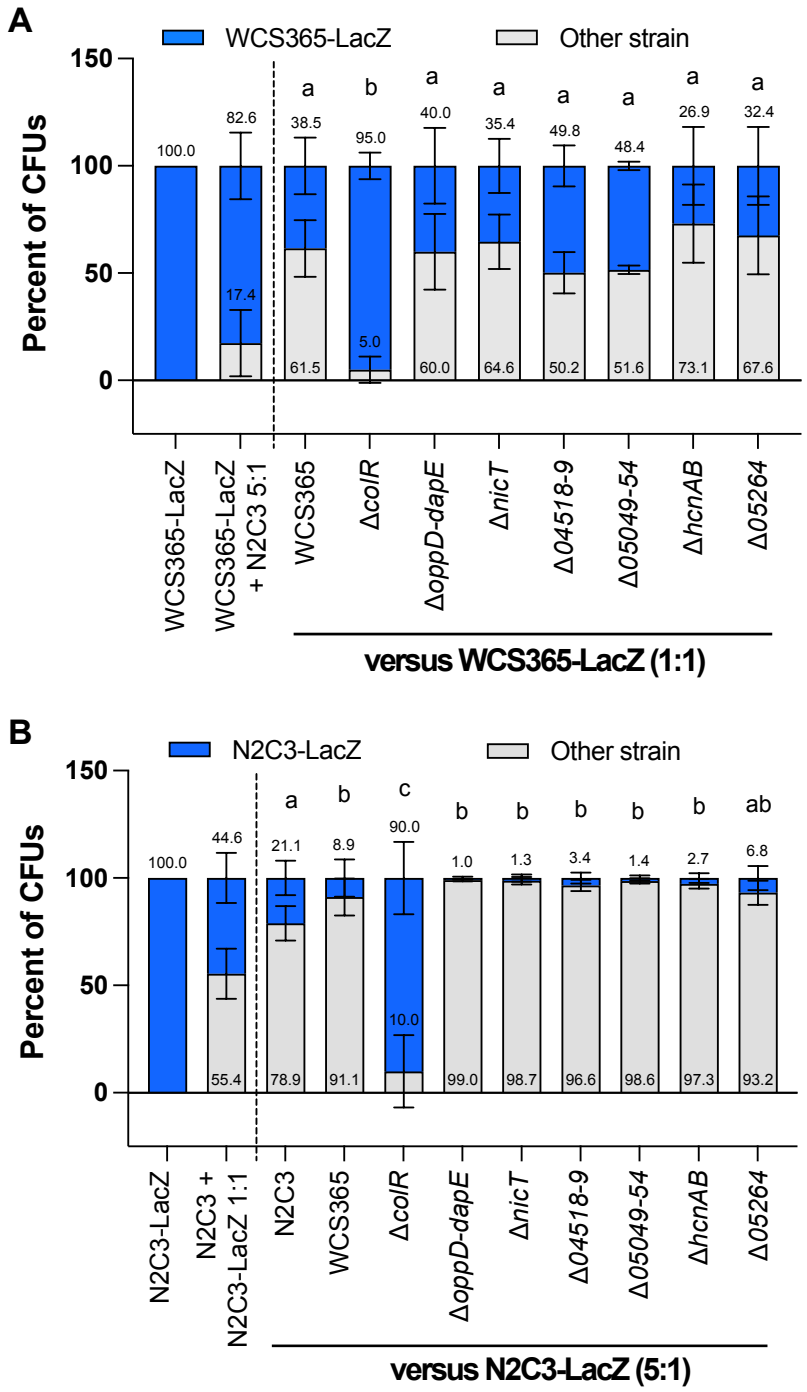


Supplemental Figure 2 LacZ reporter assay protocol. Bacteria and 7-day old *Arabidopsis* seedlings were inoculated as described in the Axenic Root Inoculation Assay protocol. Either N2C3 or WCS365 strains containing a *pMini-Tn5-lacZ* insertion were used to co-inoculate seedlings. Replicates of treatments were prepared for each timepoint (ex. 7 days post inoculation). For each timepoint, seedlings were pooled and sterilely transferred into a 2 mL microcentrifuge tube containing a metal bead and 500 µL of 10 mM $MgSO_4$. Fresh weights were measured for each treatment. Plant cells were homogenized using a Qiagen TissueLyser II at 30 Hz for 2 min. Tissue lysate was serially diluted and plated onto LB agar supplemented with 0.2 mg/mL 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal). Plates were incubated at 28°C for 2 days. Blue and white CFUs were counted, to calculate the ratio of blue LacZ-containing cells and white co-inoculated cells in the rhizosphere. Figure created with Biorender.com.

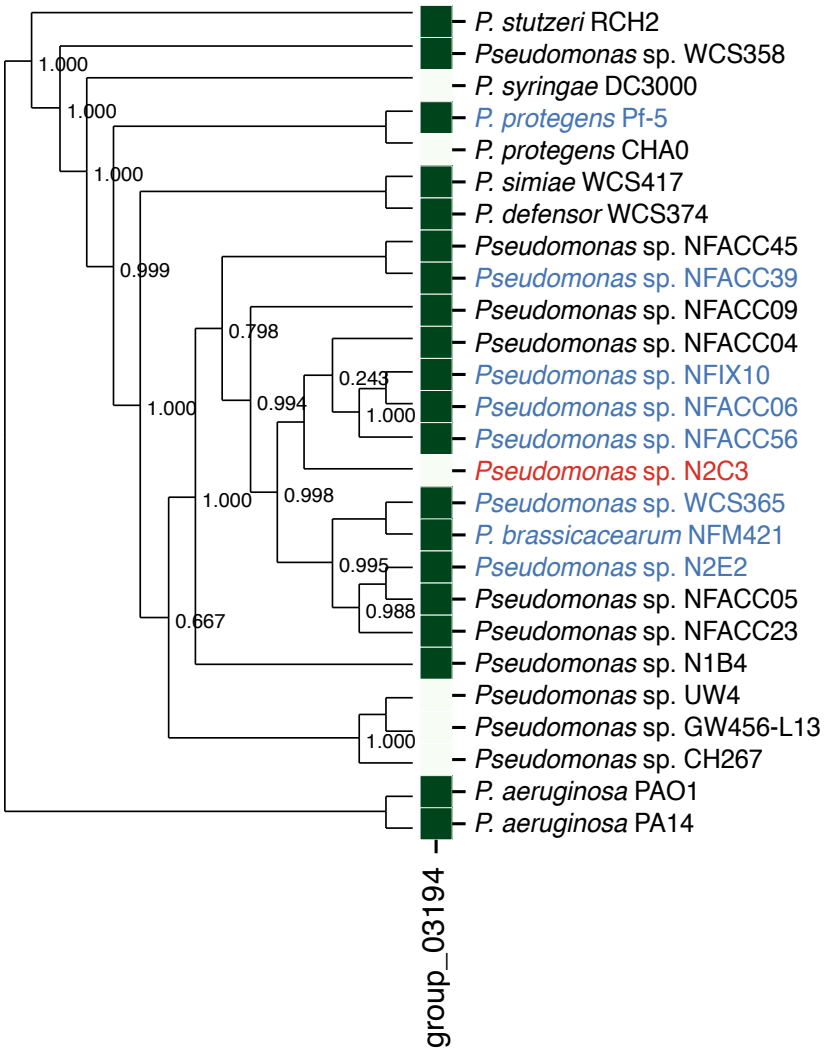
Supplemental Table 2 Primers used to generate WCS365 mutants from comparative genomics analyses.

Deletion Strain	Primer Type	Primer Sequence (5' ⟶ 3')	RE Site
WCS365 Δ*nicT*	Upstream forward	CATCAAGCTTCAGCAGCAGCGCTATGACTT	HindIII
	Upstream reverse	AGGATCAGTCCACCGCTGACGCAACTGCTCGGAGATGGTT	
	Downstream forward	AACCATCTCCGAGCAGTTGCGTCAGCGGTGGACTGATCCT	
	Downstream reverse	CATCGGATCCAATTGCCGGTAGACCTGTGC	BamHI
	Upstream confirmation	ATCCTGCAGATCAAGCAGCG	
	Downstream confirmation	TGCGCGATCTCTTCAGTGTG	
WCS365 Δ*hcnAB*	Upstream forward	CATCAAGCTTGCGAAGCCTGCGAACTGATC	HindIII
	Upstream reverse	GCTATGGCGCAGTGTCAGTCAAACAAGTCAGGCATGGGCC	
	Downstream forward	GGCCCATGCCTGACTTGTTTGACTGACACTGCGCCATAGC	
	Downstream reverse	CATCGGATCCCGCCCCATCAGGGAACAAGA	BamHI
	Upstream confirmation	TGATGGATTGGCCCTGTGCC	
	Downstream confirmation	GCCGATGATGTCATCGAGCT	
WCS365 Δ*oppD-dapE*	Upstream forward	tcatGGATCCCCGATGTGCTCAACGCACTG	BamHI
	Upstream reverse	CCCGCCTTGTCATAGCTGTTGCACCAGAATCATCAGGGCG	
	Downstream forward	CGCCCTGATGATTCTGGTGCAACAGCTATGACAAGGCGGG	
	Downstream reverse	catgAAGCTTGTTCGCTGTGACAGGCTTCC	HindIII
	Upstream confirmation	CGCGTGTTGTTCAAGCATGC	
	Downstream confirmation	ACGCTCGGTATGAACCTTGC	

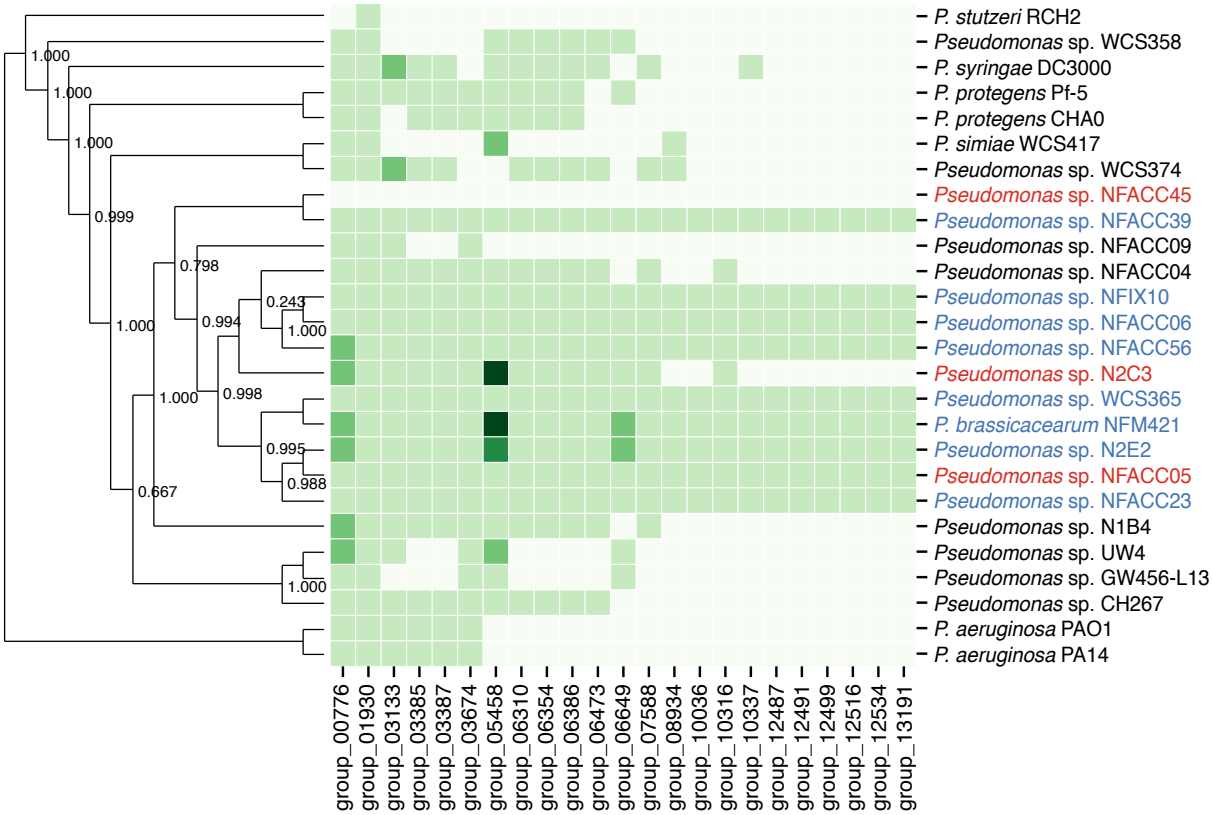
Deletion Strain	Primer Type	Primer Sequence (5' ⟶ 3')	RE Site
WCS365 Δ*04518-9*	Upstream forward	tcatGGATCCCATCCTTGGCGATCCGGTTT	BamHI
	Upstream reverse	CCAGCCACCCTCAATGTTGCCCATTGGCGTTCAATGGCAG	
	Downstream forward	CTGCCATTGAACGCCAATGGGCAACATTGAGGGTGGCTGG	
	Downstream reverse	gcatAAGCTTGAAAGTGGCTGGGACTTCAGC	HindIII
	Upstream confirmation	GTCGAGCCGTCGACTGAAAC	
	Downstream confirmation	CCTCGACAGGAATCCTGGCT	
WCS365 Δ*05049-54*	Upstream forward	tcatGGATCCCGATTATCACTGGCCGCGTC	BamHI
	Upstream reverse	AAACATCCGGATCAACGCCCATGGTGATGTTCTTGCCGCT	
	Downstream forward	AGCGGCAAGAACATCACCATGGGCGTTGATCCGGATGTTT	
	Downstream reverse	gcatAAGCTTTAGCTCGCCTCTGAAGAGGC	HindIII
	Upstream confirmation	CTGGGATCGCCATGACCAGT	
	Downstream confirmation	AGCGTGATCTGGATATCGGCT	
WCS365 Δ*05264*	Upstream forward	gcatAAGCTTGACAATCTTGGCGCGAGTCC	HindIII
	Upstream reverse	CTAGTCCGGCGATGCTATCCCAGGACACGCCAGTCTGTTG	
	Downstream forward	CAACAGACTGGCGTGTCCTGGGATAGCATCGCCGGACTAG	
	Downstream reverse	tcatGGATCCCGGCATCGAAGTAGGCGTAG	BamHI
	Upstream confirmation	CTCAATCGGATGGGCCATCAA	
	Downstream confirmation	CGACGGCTTCGTCGTTGATC	



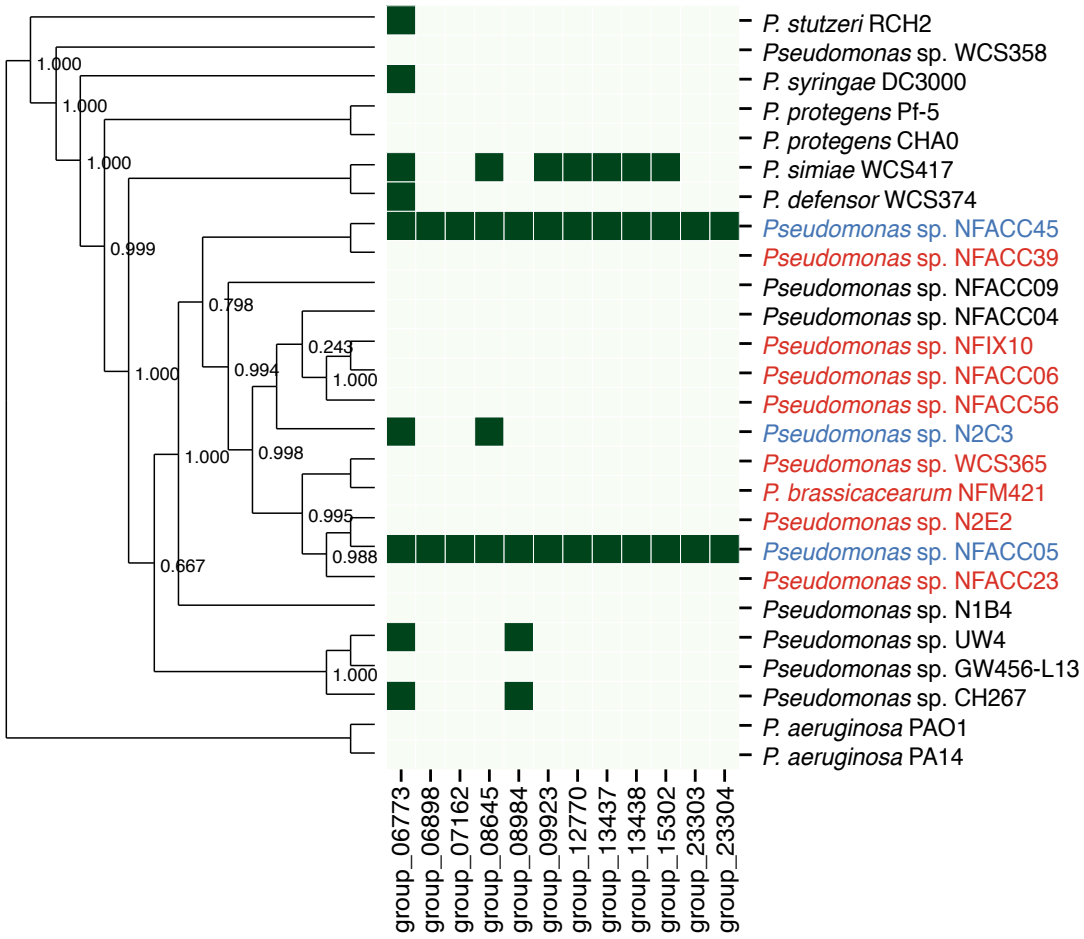
Supplemental Figure 3 Genes common to BCM strains and Pf-5 do not impact the ability of WCS365 to protect against N2C3. (A) Six WCS365 deletion mutants in genes common to BCM strains and Pf-5 were screened for rhizosphere colonization levels when co-inoculated with N2C3 at a 5:1 ratio. All of these strains were able to significantly outcompete N2C3. (B) The WCS365 deletion mutants were also tested for rhizosphere fitness defects in competition with a WCS365-LacZ strain. None of these strains had any significant competition defect in the rhizosphere.



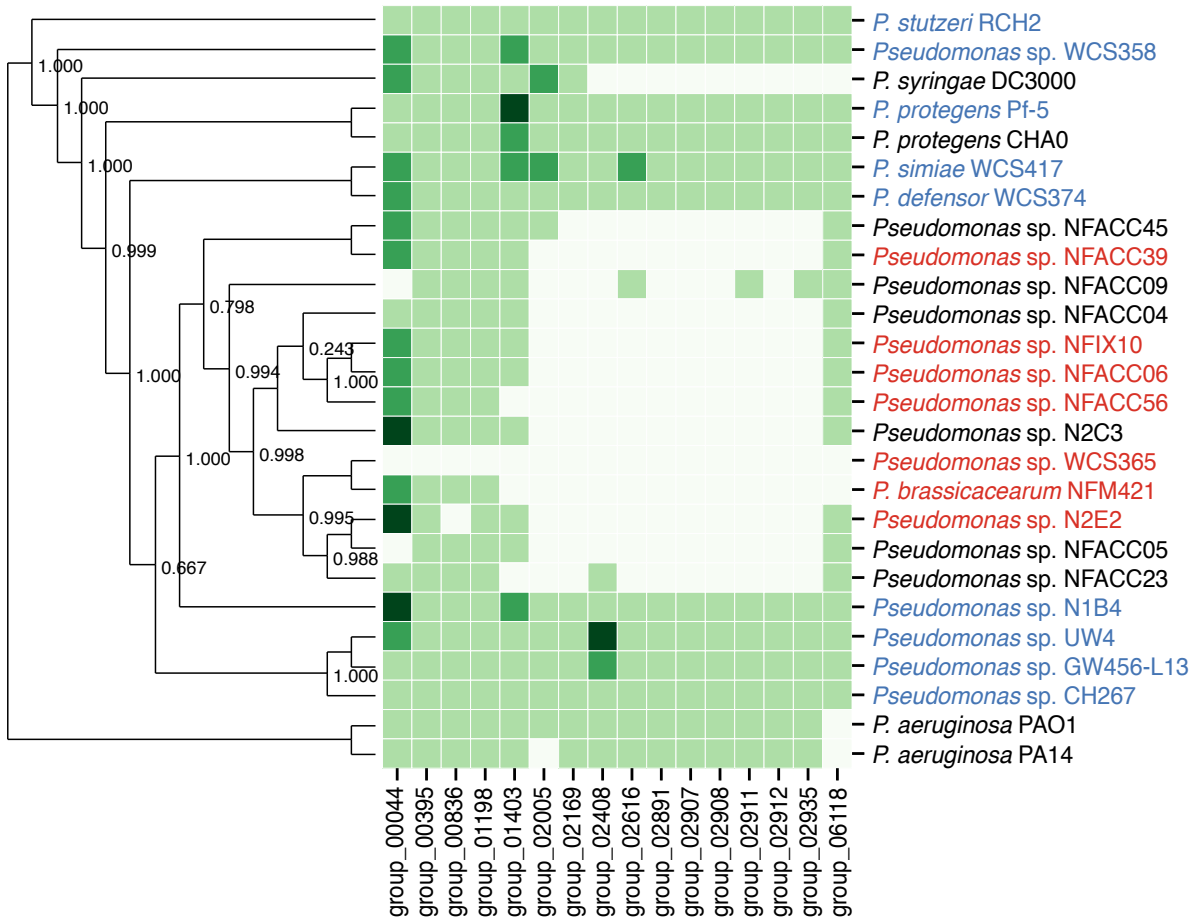
Supplemental Figure 4 Comparative genomics identified one gene unique to protective BCM strains and Pf-5. Using PyParanoid, I searched for genes present in seven protective BCM strains (NFM421, N2E2, WCS365, NFIX10, NFACC06, NFACC39, and NFACC56) and the protective non-BCM strain Pf-5 (blue labels), but absent in N2C3 (red label). This approach yielded only 1 unique gene (group_03914), encoding a CinA family protein. However, this gene was subsequently found to be present in 9 non-protective strains (NFACC05, NFACC45, N1B4, PA14, PAO1, RCH2, WCS358, WCS374, and WCS417) and absent in the protective strain CHA0, indicating that it is unlikely to underlie protection.



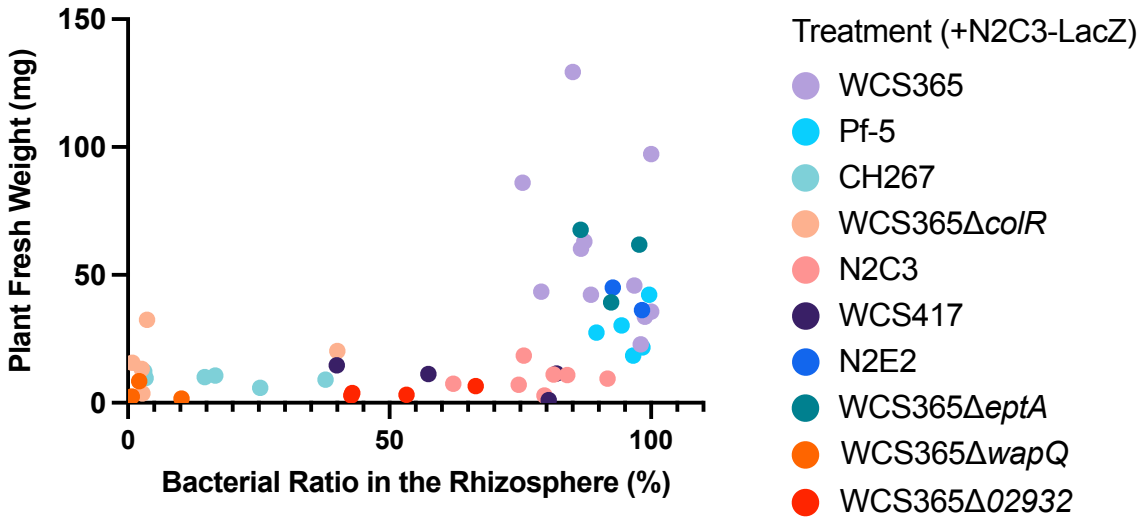
Supplemental Figure 5 A comparative genomics analysis between strains within the BCM clade yielded no genes unique to protective BCM strains. I searched for genes present within 8 protective BCM strains (NFM421, N2E2, WCS365, NFIX10, NFACC23, NFAC45, NFACC39, NFACC06, and NFACC56) (blue labels) but absent in 3 non-protective BCM strains (NFACC45, NFACC05, and N2C3) (red labels). Using this approach, I identified no genes present in the protective BCM strains, but absent in all 3 non-protective BCM strains.



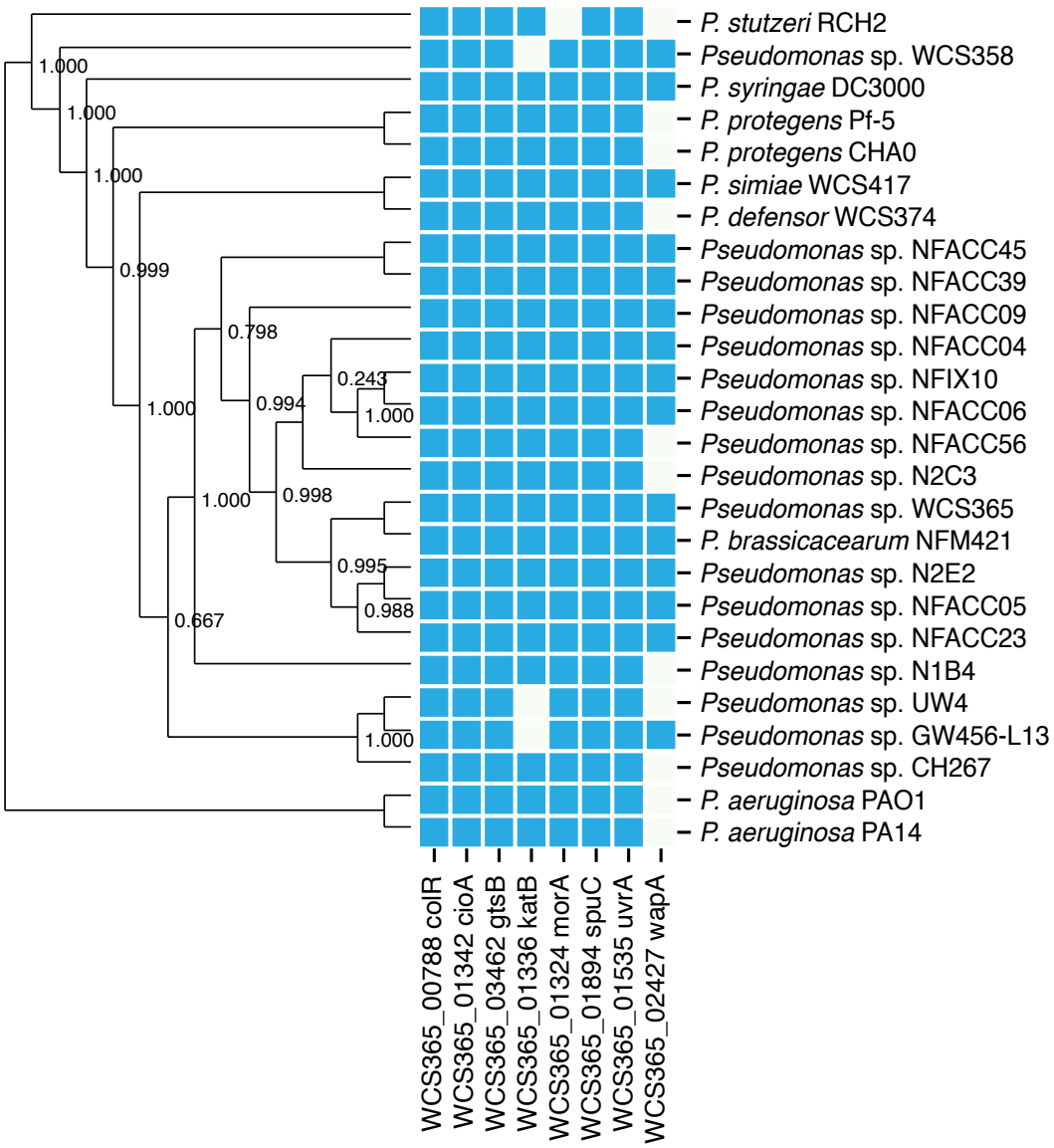
Supplemental Figure 6 Comparative genomics identified one gene unique to non-protective BCM strains. To identify genes that are unique to non-protective BCM strains, I searched for genes that were present (blue labels) in 3 non-protective BCM strains (NFACC45, NFACC05, and N2C3), but absent (red labels) in 8 protective BCM strains (NFM421, N2E2, WCS365, NFIX10, NFACC23, NFAC45, NFACC39, NFACC06, and NFACC56). Using this approach, I identified 1 gene group encoding an aldo/keto reductase (group_06773) that was unique to non-protective BCM strains. However, not all non-protective strains outside of the BCM clade possessed this gene group, indicating that the absence of this gene was unlikely to be responsible for protection in the BCM clade.



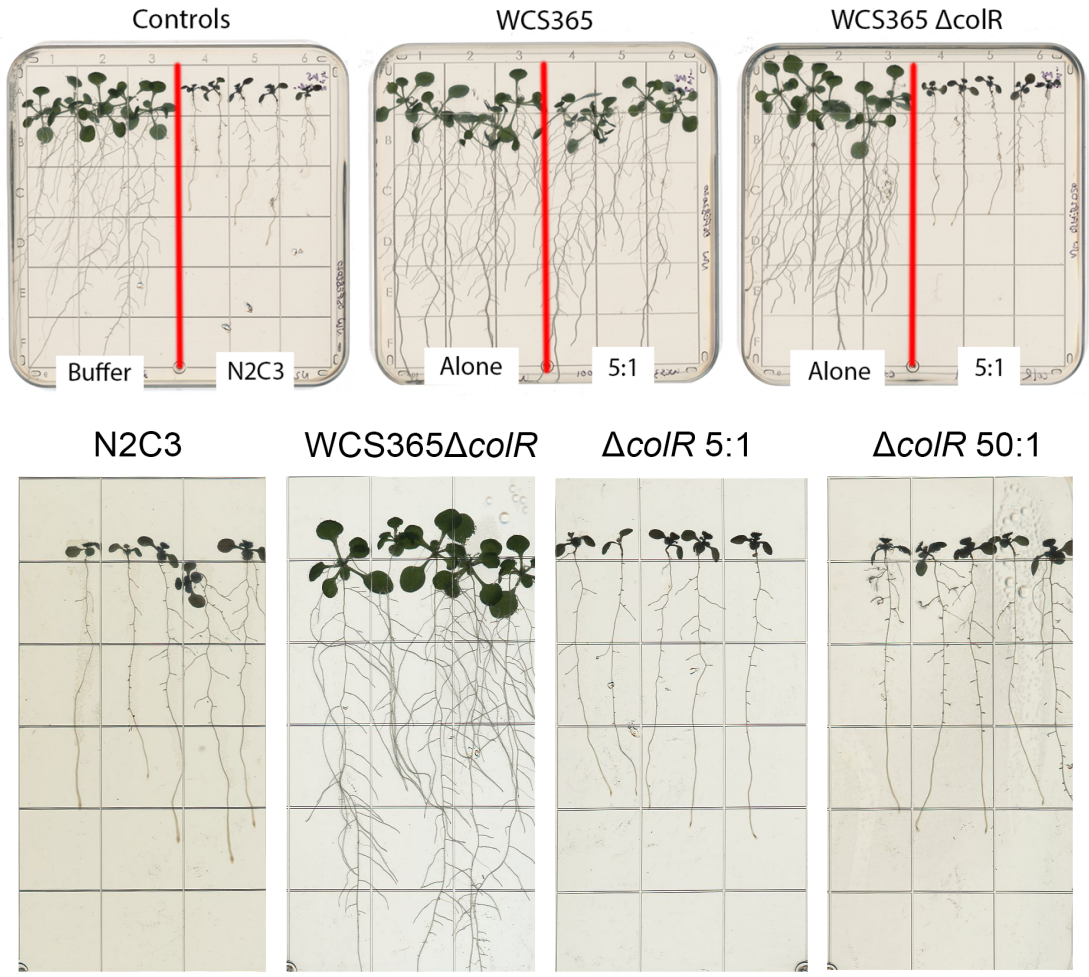
Supplemental Figure 7 Comparative genomics failed to identify genes absent only in protective BCM strains. Based on my hypothesis that Pf-5 uses a different mechanism for protection, I searched for genes whose absence may be responsible for a BCM clade-specific mechanism of protection. Comparative genomics was used to identify genes present in 8 non-protective strains (RCH2, WCS358, WCS417, WCS374, N1B4, UW4, GW456-L13, and CH267) (blue labels) and Pf-5, but absent in 7 protective BCM strains (NFACC39, NFIX10, NFACC06, NFACC56, WCS365, NFM421, and N2E2) (red labels). I was unable to find any gene groups that met this criterion.



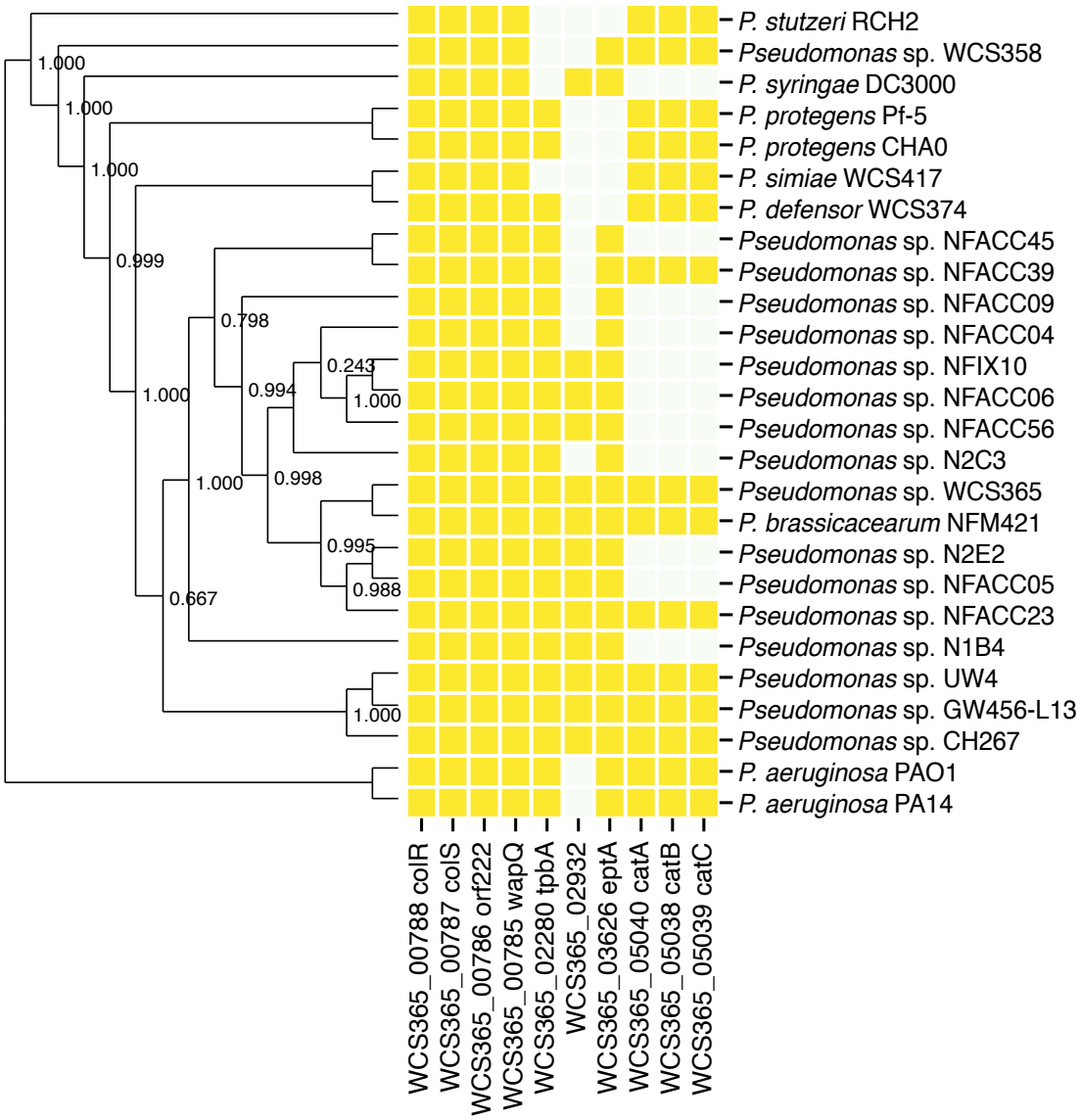
Supplemental Figure 8 Protection against *Pseudomonas* sp. N2C3 is rhizosphere colonization dependent. Correlation between the bacterial fitness in the rhizosphere and plant fresh weight. Data points are colour-coded by treatment, and represent the averaged fresh weights for individual biological replicates from the data shown in Figure 2.8 and Figure 2.11. Each strain was co-inoculated with N2C3-LacZ at a 5:1 ratio.



Supplemental Figure 9 Distribution of colonization genes across *Pseudomonas* spp. Previously identified genes involved in colonization and rhizosphere fitness (25) are largely conserved across both protective and non-protective *Pseudomonas* strains.



Supplemental Figure 10 Representative images showing loss of protection by *Pseudomonas* sp. WCS365 Δ*colR* mutant. WCS365 and WCS365Δ*colR* were compared for their ability to protect *Arabidopsis* against N2C3. Ratios indicate the volume of the test strain:N2C3. In comparison to plants co-inoculated with WCS365 and N2C3, plants co-inoculated with WCS365Δ*colR* and N2C3 were more stunted, indicating that deletion of *colR* negatively impacts the ability of WCS365 to protect *Arabidopsis* from N2C3**.**



Supplemental Figure 11 Distribution of ColR-dependent genes across *Pseudomonas* spp. Genes whose expression was identified to be regulated by ColR (C.L. Wiesmann et al., unpublished data) are largely conserved across both protective and non-protective *Pseudomonas* strains.