R-TAILOCIN PRODUCTION AND BIOLOGICAL FUNCTION IN THE PLANT GROWTH PROMOTING RHIZOBACTERIA, *PSEUDOMONAS CHLORORAPHIS*

A Dissertation

by

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ABSTRACT

R-tailocins are bacteriocins resembling contractile bacteriophage tails. Recent studies identified R-tailocin gene clusters within several plant growth promoting (PGPR) *Pseudomonas* genomes, but the ecological significance of these R-tailocins remains unknown. In this study, we analyzed sequenced PGPR *Pseudomonas* genomes and found that the R-tailocin gene cluster within most *P. chlororaphis* and some *P. fluorescens* strains contain two R-tailocin genetic modules. Phylogenetic analysis revealed that these genetic modules may have different ancestral origins. Beyond genomic analysis, we demonstrate that the PGPR, *P. chlororaphis* 30-84, produces two distinct R-tailocin particles, each with a discrete killing spectrum. Further study found that *P. chlororaphis* 30-84 not only produces two distinct tailocin particles, but that one of them is produced with two different types of tail fibers that widen its killing spectrum. The killing spectrum of tailocin 1 is limited to *Pseudomonas* whereas tailocin 2 targets both *Pseudomonas* and *Xanthomonas* strains. The spectra of pseudomonads killed by the two R-tailocins differed, although a few *Pseudomonas* species were either killed by or insusceptible to both. Tailocin release was disrupted by deletion of the holin gene within the tailocin gene cluster, demonstrating that the lysis cassette is required for the release of both R-tailocins. The loss of functional tailocin production reduced the ability of *P. chlororaphis* 30-84 to compete with an R-tailocin-sensitive strain within biofilms and rhizosphere communities. The breadth of influence of the R-tailocins on native wheat rhizobacteria was examined by screening a collection of wheat

rhizobacteria (484 strains) for susceptibility to either tailocin. Nearly 12% of the collection was targeted by one of the tailocins, the majority being susceptible to tailocin 2. Loss of production of one or both tailocins resulted in decreased *P. chlororaphis* 30-84 persistence within the wheat rhizosphere when in competition with the native microflora, but not bulk soil. PGPR need to survive and persist within complex and dynamic rhizosphere microbial communities. This study demonstrates that *P. chlororaphis* possess a unique R-tailocin gene cluster and that R-tailocins serve as a competitive mechanism that enables *P. chlororaphis* persistence within the rhizopshere microbial community. R-tailocin production should be considered when vetting PGPR for inoculants.

DEDICATION

To my parents, Cynthia and Walter, for all their love, encouragement, and support

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CHAPTER I

GENERAL INTRODUCTION

Bacteria naturally exist in complex biofilm communities where competition for space and resources is high (1-3). Bacteria employ interference strategies to compete for space and nutrients in these communities, including the production of bacteriocins (3-7). These are bactericidal proteins that generally target bacteria closely related to the producer, affording the producer a competitive advantage over strains sensitive to the specific bacteriocin produced. There are two classes of bacteriocins, low and high molecular weight (6, 8).

Phage tail-like bacteriocins, or tailocins, are high molecular weight bactericidal protein complexes that resemble and are evolutionarily related to bacteriophage tails (9, 10). There are two structurally different classes of tailocin particles, the F- and R-type (9, 11). F-type tailocins are flexible, non-contractile particles that resemble and are evolutionarily related to *Siphoviridae* bacteriophage tails (9, 11-13). R-type tailocins are rigid, contractile particles that are evolutionarily related to and resemble *Myoviridae* bacteriophage tails (9, 11, 12). R-Tailocins are produced by a diversity of *Bacteria* (Gram-negative and Gram-positive), including; *Pseudomonas aeruginosa* (11), *Pseudomonas putida* (14), *Pseudomonas syringae* (15-18), *Pseudomonas fluorescens* (19), *Burkholderia cenocepacia* (10), *Clostridium difficile* (20), *Vibrio cholera* (21), *Erwinia carotovora* (22), *Rhizobium lupine* (23), *Serratia plymithicum* (24), *Serratia*

maltophilia (25)*, Xenorhabdus nematophilus* (26)*, Xenorhabdus bovienii* (27)*,* and *Proteus vulgaris* (28). Much of the research exploring the production and physical properties of R-tailocins has concentrated on tailocins produced by human pathogenic bacteria, such as *P. aeruginosa,* because of their potential as alternatives to broad-spectrum antibiotics (20, 29-31).

Tailocin production is chromosomally encoded within gene clusters that have similar genetic content and organization as bacteriophage tail genetic modules (9). In *Pseudomonas,* the chromosomal insertion point of the tailocin gene cluster within the bacterial chromosome varies among species, but is generally located between *trpE* and *trpG* or *mutS* and *cinA* (14, 15, 32, 33). For example, in *P. aeruginosa* and *P. syringae* the R-tailocin gene cluster is situated between *trpE* and *trpG* whereas it is located between *mutS* and *cinA* in *P. fluorescens* (15, 33). Tailocin gene clusters encode regulatory, structural, and lysis cassette genes, the latter mediate the extracellular release of tailocins (9, 11).

The regulation of tailocin production is associated with induction of the SOS response and tailocin synthesis is universally triggered by the application of DNA damaging agents, such as ultraviolet light and mitomycin C (9, 15, 34). The regulatory genes governing tailocin production were first identified and characterized in *P. aeruginosa*. *P. aeruginosa* tailocin production is regulated by a repressor (PrtR), an activator (PrtN), and the DNA repair and homologous recombination enabling protein (RecA). In the absence of DNA damage, PrtR represses the expression of *prtN*. Upon DNA damage, activation of RecA results in the cleavage of PrtR and production of PrtN,

which induces the expression of the tailocin structural and lysis genes (34). Interestingly, the regulatory elements encoded within the R-tailocin gene clusters vary among *Pseudomonas* strains, suggesting differences in the regulation of R-tailocin synthesis and release (14). For example, *P. fluorescens* and *P. putida* strains lack a homolog of *prtN* found in *P. aeruginosa* (14).

The structure of the R-tailocin produced by *P. aeruginosa* has been studied extensively (35-41). This particle consists of a double hollow cylinder that is composed of a sheath and inner core (Fig. 1.1). The length of the sheath-core cylinder is determined by the tape measure protein (42). Attached to the distal end of the core is a pointed tail spike protein that facilitates the puncturing of susceptible bacterial cell walls (9, 43). The inside of the core has a negative charge, which may mediate the translocation of positively charged ions into susceptible cells after contraction (41). The sheath-core cylinder is connected to a baseplate that has six tail fiber proteins attached to it (9, 44). The tail fibers function as receptor binding proteins, which interact with lipopolysaccharide or lipooligosaccharide receptors of susceptible cells (14, 45, 46). Approximately 100-200 R-tailocins are synthesized in each *P. aeruginosa* cell (44), whereas a similar study found an average of 600 tailocin particles per cell in *Burkholderia cenocepacia* (10).

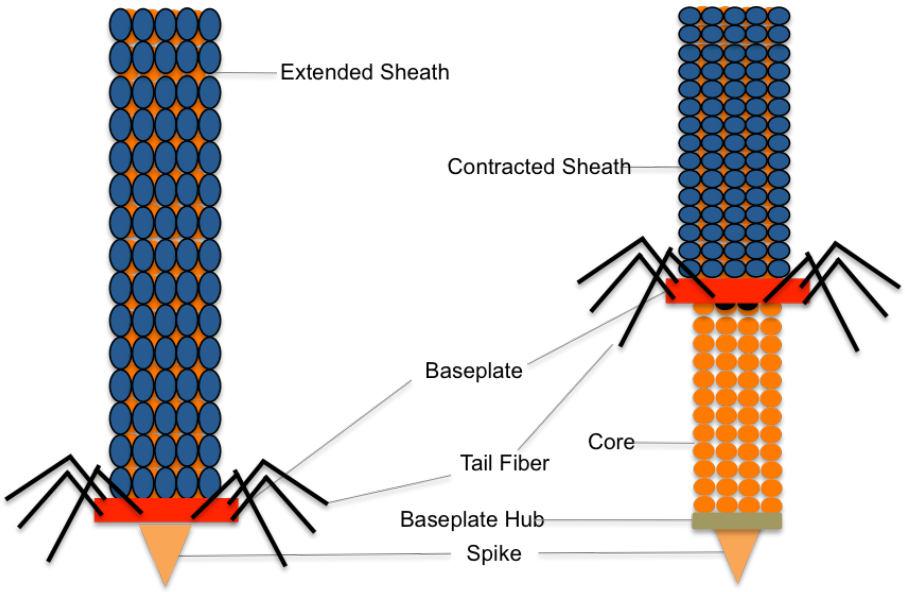

Figure 1.1. R-tailocin Structure. Displayed are the extended (left) and contracted (right) R-tailocin particle structures. The R-tailocin particle consists of a core (orange) that is enclosed within a contractible sheath (blue). Tail fibers (black) are attached to the baseplate (red), and a tail spike (light orange) is connected to the central core via the baseplate hub (tan).

The extracellular release of tailocin particles is mediated by cell lysis, which is facilitated by a lysis cassette that is similar to those found in lytic bacteriophage (12, 47). In tailocin gene clusters, the lysis gene cassette may consist of a holin, cytoplasmic endolysin, and spanin complex (Fig. 1.2). Some tailocin gene clusters include genes encoding all three components, whereas others (e.g., *P. aeruginosa*), do not contain the gene for a spanin complex (14). Holins are small transmembrane proteins that accumulate in the inner membrane and form pores when a threshold of holin molecules is accrued in the membrane. Endolysins are proteins that catalyze the degradation of the peptidoglycan layer and are generally unable to cross the inner membrane without the

aid of a holin. The holin mediated inner membrane pores provide the endolysin access to degrade the peptidoglycan layer (48). The spanin complex is a membrane-spanning complex that is responsible for disruption of the outer membrane in Gram negative bacteria (49).

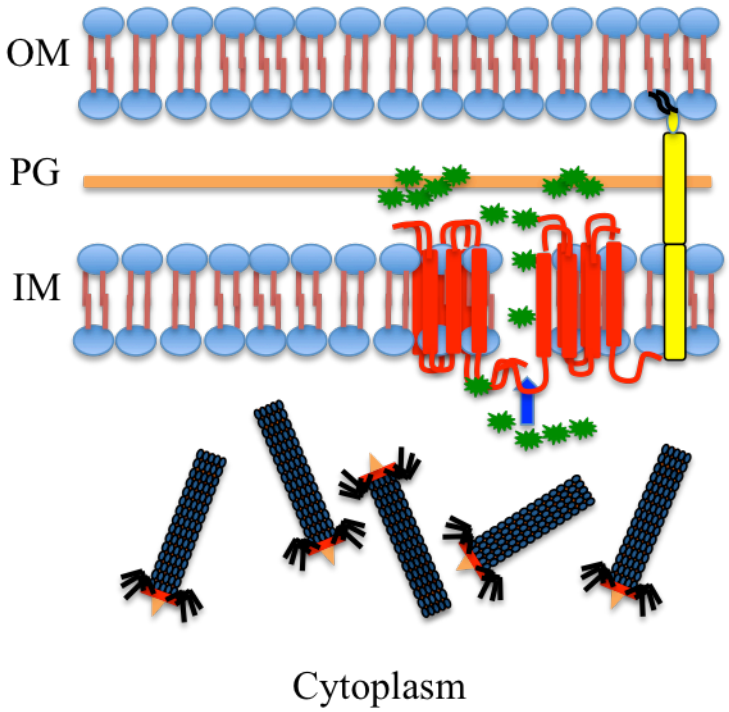

Figure 1.2. R-tailocin and bacteriophage lysis cassette. The lysis cassette is composed of the holin (red), endolysin (green), and spanin complex (yellow). The holins accumulate in the inner membrane and form a channel through which the endolysin travels. In the periplasmic space, the endolysin breaks down the peptidoglycan layer. The spanin complex disrupts the outer membrane. The combined activity of the lysis cassette components results in cell lysis and tailocin release.

Upon release, the R-tailocin tail fibers interact with surface-bound receptors of susceptible bacterial cells, generally lipopolysaccharides, which triggers contraction of the tail spike capped core through the bacterial cell-wall (38). This creates a channel through which positively charged ions travel, which subsequently depolarizes the membrane potential, halting respiration (Fig. 1.3). The formation of these pores in the membrane also halts macromolecular synthesis, causes the release of cytoplasmic content and cell shrinkage, and eventually results in death and lysis of the tailocin-sensitive cell (39, 41, 50). Tailocin-mediated cell death is an extremely efficient process, requiring only one tailocin particle to kill a susceptible cell (10).

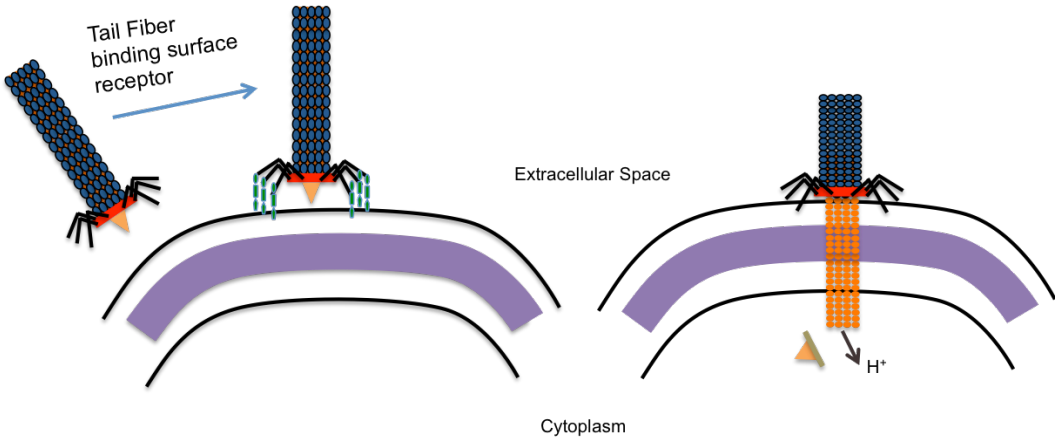

Figure 1.3. R-Tailocin killing mechanism. R-tailocin particles tail fibers bind to surface-bound receptors (outer core of LPS, teal) of susceptible cells (left). Upon receptor binding, the sheath contracts and forces the inner core through the membrane of susceptible cells, which opens a channel in the membrane that disrupts ion gradients and cellular respiration, leading to cell death (right).

Given their potent antibacterial activity, it is not surprising R-tailocins have been shown to play a role in competition with other bacteria, although to date, most competition studies have been limited to pairwise comparisons of tailocin producers and isolates collected from the same niche. For example, R-tailocin production by *P. aeruginosa* facilitates a growth advantage over sensitive strains in pairwise co-culture and biofilm experiments and influences population dynamics in tracheal aspirates (45, 51-53). Similar studies in the nematode mutualists, *X. nematophila* and *X. bovienii*, found that R-tailocin production gives it a competitive advantage over sensitive strains in the nematode gut and co-culture experiments (26, 27). Little is known about the broader role of tailocin production in the survival of these producing strains in natural microbial communities.

Recent genomic studies revealed that tailocin gene clusters are relatively common in the genomes of plant-associated *Pseudomonas* species, both plant pathogenic and plant-beneficial isolates (14, 15, 32, 33). Phylogenetic analysis of these gene clusters categorized the R-tailocins into four groups based on evolutionary relationships and the complex of species having each type of gene cluster. These groups, Rp1, Rp2, Rp3, and Rp4, are represented by tailocins produced by *P. aeruginosa*, *P. putida*, *P. fluorescens,* and *P. syringae*, respectively (14, 15). Most of the strains analyzed are predicted to produce R-tailocins, however a few *P. fluorescens* strains are postulated to produce two R-tailocins or a R-tailocin and F-tailocins (33). In addition to the genomic studies, the R-tailocins produced by several plant-associated *Pseudomonas* have been characterized

(14-16, 18). The known host range of these tailocins is generally limited to members of *Pseudomonas* with the exception of a tailocin produced by *P. fluorescens* SF4c, which also targets plant-associated *Xanthomonas* species (50). Similar to the R-tailocins produced by *P. aeruginosa* (45) and *B. cenocepacia* (10), the receptor targeted by plant-associated *Pseudomonas* species is related to the lipopolysaccharides on the outer membrane (14, 54). The extensive prevalence of R-tailocin gene clusters within the genomes of plant-associated *Pseudomonas* as well as the recent work characterizing R-tailocins produced by some of them suggest that R-tailocins serve an important function in the ecology of plant-associated bacterial communities. To date, no research has explored the relative importance of these particles in mediating the population dynamics of plant-associated bacterial populations.

Similar to the human microbiome, the plant microbiome constitutes a 'second plant genome' capable of enhancing plant health (55). Plants actively recruit a rhizosphere microbiome from the surrounding bulk soil with plant-derived signals and root exudates that enable microbial recognition and directional motility, as well as the ordered development of plant-associated communities (55-58). An important subset of the rhizosphere microbiome consists of plant growth promoting rhizobacteria (PGPR) that facilitate nutrient uptake, protect against stress or pathogens, and contribute to plant growth and fitness (55, 59-62). The plant rhizosphere is a complex landscape comprised of dynamic microbial niches inhabited by diverse microbial communities with which introduced PGPR must effectively compete in order to successfully establish populations and bring about desired benefits (59, 60). The abundance of microbes competing for

limited rhizosphere resources ostensibly necessitates the use of multiple competitive survival strategies. In order to maximize the plant-PGPR interaction to enhance plant health, a better understanding of the mechanisms governing competitive interactions and PGPR persistence in the rhizosphere is required (63, 64).

Fluorescent pseudomonads are among the most ubiquitous and conspicuous inhabitants of the rhizosphere microbiome (65, 66). These organisms produce a vast array of antimicrobial secondary metabolites that contribute to biological control of plant pathogens as well as phytohormones that modulate plant growth, responses to pathogen attack, and abiotic stress (61, 67-71). *P. chlororaphis* subsp. *aureofaciens* 30-84 is a PGPR characterized for its use as a biological control of take-all disease of wheat. This strain produces phenazine antibiotics that are required for disease suppression (72). In addition, phenazines contribute to *P. chlororaphis* 30-84 biofilm formation and rhizosphere fitness, and have broad-reaching influence on gene expression patterns (73-75). Production of the phenazine 2-hydroxy-phenazine-1-carboxylic acid by *P. chlororaphis* 30-84 induces the expression of a R-tailocin resembling gene cluster (73). Preliminary analysis of the *P. chlororaphis* 30-84 R-tailocin gene cluster revealed features that had not been reported or characterized in the R-tailocin gene clusters of the *Pseudomonas* species described previously (15). The most conspicuous feature of the *P. chlororaphis* 30-84 tailocin gene cluster is that it contains two R-tailocin structural genetic modules. Another interesting finding was the presence of only one lysis cassette for the entire R-tailocin cluster, suggesting that if both of the distinct genetic modules encode functional R-tailocins, they are produced and released together. As reported in

Chapter II, these novel features are found in the genomes of most sequenced *P. chlororaphis* strains, but are *not common in the genomes of other sequenced Pseudomonas* spp. The significance of these observations (and subsequent observations of multiple tail fiber genes within the tailocin 2 gene cluster) for R-tailocin production by *P. chlororaphis* 30-84 and the relative importance of R-tailocin production for the fitness of this plant-associated PGPR in the rhizosphere are the broad subjects of my dissertation.

This dissertation is divided into two main research chapters, a discussion chapter, and an appendix. The broad goals of the first research chapter (chapter II) were to characterize *P. chlororaphis* 30-84 tailocin production and describe the biological role of tailocin production for *P. chlororaphis* 30-84 in pairwise interactions in biofilms and on wheat roots. The specific objectives of the first research chapter were to:

1) Examine the phylogenetic relationships between the *P. chlororaphis* 30-84 tailocin structural genetic modules and those encoded in the genomes of other *Pseudomonas* species as well as known bacteriophages.
2) Analyze the physical characteristics of the tailocins produced
3) Determine the host range of the tailocin(s) produced.
4) Functionally characterize the lysis cassette associated with the R-tailocin gene cluster and determine its role in R-tailocin release.
5) Determine whether R-tailocin production affords *P. chlororaphis* 30-84 a competitive advantage over a sensitive rhizosphere strain in pairwise

competition assays when grown in surface attached biofilms and wheat roots.

Chapter III expands on the findings revealed during the experiments discussed in Chapter II. For example, the examination of the *P. chlororaphis* 30-84 R-tailocin gene cluster in Chapter II also revealed the presence of two putative tail fiber genes in the tailocin 2 gene module. If both were found to be functional, this could constitute a previously undescribed mechanism for conferring a broader killing spectrum to a R-tailocin. As reported in Chapter III, this novel feature is found in the tailocin 2-like genetic modules of most sequenced *P. chlororaphis* and some *P. fluorescens* strains. Additionally, studies exploring the ecological function of R-tailocins in Chapter II were confined to the study of pairwise interactions. What remained to be determined for *P. chlororaphis* 30-84 and for other R-tailocin producers in general is an understanding of the relative importance of R-tailocin production for competitive survival within complex communities of microorganisms, such as those found in the natural wheat rhizosphere microbiome. In chapter III, we utilize *P. chlororaphis* 30-84 and R-tailocin deficient derivative strains to examine the role of R-tailocin production in *P. chlororaphis* 30-84 persistence in the native rhizosphere and bulk soil microbiomes. The specific objectives of the second main chapter were to:

1) Characterize the function of the two tail fibers identified within the tailocin 2 genetic module. More specifically, we address the following questions:

a. Do the tail fibers function independently of one another or are both required for function (killing)?
b. Are the tail fibers attached to the same tailocin particle (e.g. resulting in a chimeric particle with two tail fibers) or are they incorporated onto separate tailocin particles?
c. If they incorporate separately, does each type of tailocin 2 particle have the same killing spectrum?

2) Estimate the breadth of influence of tailocin production on the diversity of microbes found in the rhizosphere by screening a collection of culturable bacteria isolated from the wheat rhizosphere to determine the proportion that is targeted by each of the tailocins produced by *P. chlororaphis* 30-84.
3) Examine the role of R-tailocin production in the persistence of *P. chlororaphis* 30-84 in competition with the community of organisms in the wheat root-rhizosphere and in bulk soil.

Chapter IV- *Conclusions* Summarizes the significance of my work not only in characterizing the R-tailocin gene cluster and R-tailocin production in *P. chlororaphis* 30-84, but relative to other plant-associated pseudomonads. My work also demonstrates the importance of R-tailocin production not merely for competition with culturable rhizosphere isolates, but for competitive survival and persistence of this PGPR in the rhizosphere. Perhaps this R-tailocin production is a strategy that should be utilized when considering PGPR traits. I discuss these findings within the context of rhizosphere community dynamics and contrast them with observations on the importance of R-

tailocins in bulk soil, discussing factors that may limit the utility of R-tailocins as a competitive strategy in bulk soil. I conclude with observations on the significance of my work and possible future directions for this research.

The appendix includes my work on a project that is unrelated to the main chapters of my dissertation. Early in my graduate experience, I contributed to a project focused on the genetic determinants of small colony variants of *P. chlororaphis* 30-84 that commonly emerge from static biofilms. This work culminated in the following publication: Wang D, Dorosky RJ, Han CS, Lo C, Dichosa AEK, Chain PS, Yu JM, Pierson LS, III, Pierson EA. 2015. Adaptation genomics of a small-colony variant in a *Pseudomonas chlororaphis* 30-84 biofilm. *Appl Environ Microbiol* 81:890–899. doi:10.1128/AEM.02617-14. In this appendix, I provide a brief introduction to the purpose and significant findings of the research and my contribution to the published work. I describe additional questions of interest to me and experiments that I performed that were not a part of the published work. I discuss these results in the context of how they add to the published work and possible future directions for the research.

CHAPTER II

PSEUDOMONAS CHLORORAPHIS PRODUCES TWO DISTINCT R-TAILOCINS THAT CONTRIBUTE TO BACTERIAL COMPETITION IN BIOFILMS AND ON ROOTS[1]

Introduction

Bacteria produce a diversity of bacteriocins to kill closely related competitors (3). Tailocins are high-molecular weight (HMW) bacteriocins produced by a variety of bacteria that resemble bacteriophage tails (8, 11, 20, 77). Tailocin particles are protease resistant, thermolabile, and sedimentable by ultracentrifugation (8). These particles have been studied extensively in the opportunistic human pathogen *Pseudomonas aeruginosa*, which are capable of producing either or both R-type and F-type tailocins (11). R-type tailocins are rigid rod-like particles with a contractile sheath and resemble T-even coliphage tails whereas F-type tailocins are flexible sheathless particles that resemble lambda phage tails (12).

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[1]Reprinted with permission from Dorosky RJ, Yu JM, Pierson LS, Pierson EA. 2017. *Pseudomonas chlororaphis* produces two distinct R-Tailocins that contribute to bacterial competition in biofilms and on roots. Applied and Environmental Microbiology: AEM. 00706-17.

The *P. aeruginosa* R-tailocin particle is composed of a double hollow cylinder that consists of a sheath and core connected to a baseplate with six tail fibers (36, 37). In *P. aeruginosa,* R-tailocin synthesis is induced by the SOS response and yields 100-200 tailocins per cell (34). A lysis cassette similar to that utilized by lytic bacteriophage mediates the extracellular release of R-tailocin particles (12). The bacteriophage lysis cassette is composed of genes encoding the endolysin, holin, and Rz, and Rz1 proteins comprising the spanin complex (47). Some tailocin gene clusters encode all three lysis cassette components, while others, such as those encoded in *P. aeruginosa*, generally do not encode the spanin complex (14). Endolysins are cytoplasmic proteins that catalyze the degradation of the cell wall peptidoglycan layer. Holins are small, transmembrane proteins that accumulate in the inner membrane, and permeabilize the membrane, which allows the endolysins to reach and degrade the peptidoglycan layer (48). Rz and Rz1 make the spanin complex, which is involved in disruption of the outer membrane and release of bacteriophage (49). Once released, R-type tailocin tail fibers interact with specific surface receptors (lipopolysaccharides) of susceptible bacterial competitors and insert a needle-like core through the cell wall. This depolarizes the membrane potential and stops macromolecular synthesis leading to cell death (38, 39).

Host-killing range is determined by the specificity of the interaction between the tailocin particle and the target surface receptors and is generally limited to closely related species or strains. Producing strains are typically resistant to the R-tailocin they release (11, 15, 19, 31, 40). Although host-killing ranges of some strains have been characterized, the extent to which R-tailocins contribute to the competitive dynamics of

bacterial communities is less well understood. The importance of R-tailocin production and release in interstrain interactions was investigated in *P. aeruginosa*, revealing that R-tailocin production results in a an advantage for the producing strain in mixed populations with susceptible strains *in vitro* as well as within human host tissues (45, 51, 53).

More recently, R-tailocins similar to those produced by *P. aeruginosa* have been identified in plant associated *Pseudomonas* strains (14, 15, 19, 32, 78), suggesting that R-tailocin production may be an important competitive determinant of bacterial interactions for *Pseudomonas* inhabiting diverse environments, such as the plant microbiome. In the present study, we focus on *Pseudomonas chlororaphis* 30-84, a rhizosphere-colonizing strain selected for its ability to suppress take-all disease of wheat, caused by the fungal plant pathogen *Gaeumannomyces graminis* var. *tritici.* The production of phenazines by *P. chlororaphis* 30-84 is the primary mechanism of pathogen inhibition and contributes to rhizosphere persistence as well as biofilm development (72, 74, 75, 79). Recently, production of the phenazine, 2-hydroxy-phenazine-carboxylic acid (2-OH-PCA) by *P. chlororaphis* 30-84 was shown to promote biofilm formation by the release of extracellular DNA (eDNA) (73). Increased production of eDNA in the biofilm of *P. chlororaphis* 30-84 was due in part to cell autolysis as a result of the expression of a gene cluster with sequence similarity to R-tailocin gene clusters in *P. aeruginosa* (73).

Despite the identification of R-tailocin gene clusters in plant-associated *Pseudomonas* strains, they have received little attention beyond genomic comparison

and killing spectrum studies. Previous comparison of the R-tailocin gene clusters among *Pseudomonas* strains revealed distinct evolutionary ancestries based on the protein sequences of the structural components that may be influenced by bacterial habitat (14, 15). Analysis of the *P. chlororaphis* 30-84 R-tailocin gene cluster suggested a unique evolutionary trajectory because it contains structural components with similarity to both *P. aeruginosa* and *P. syringae,* potentially representing a hybrid tailocin (15). Closer analysis, as described in this study led us to hypothesize that the cluster may encode two functional R-tailocins sharing a single lysis cassette. If the two R-tailocins had different killing spectra, this could enhance the competitive ability of *P. chlororaphis* 30-84 strain over a broader spectrum of closely related species competing for similar ecological rhizosphere niches. The aims of this study were to: (1) further characterize the *P. chlororaphis* 30-84 tailocin gene cluster in comparison to other plant-associated *Pseudomonas* species; (2) determine whether *P. chlororaphis* 30-84 produces two functional R-tailocins as predicted from genome analysis and compare the killing spectrum of each; (3) determine the functionality of the lysis cassette in R-tailocin release; and (4) evaluate the role of R-tailocin production in competition between rhizosphere associated-bacteria in surface attached biofilms and on plant roots.

Experimental Procedures

Bacterial Strains and Media. The bacterial strains and plasmids used in this study are described in Table 2.1. A spontaneous rifampin-resistant derivative of *P. chlororaphis* 30-84 was used in all studies. *P. chlororaphis* 30-84 and *P. putida* KT2440 were grown at 28°C in Luria Bertani (LB) medium or AB minimal medium supplemented with 2% Casamino Acids (AB + CAA) (80). *E. coli* strains were grown at 37°C in LB medium. Antibiotics were used when appropriate at the following concentrations: for *E. coli*, kanamycin (Km) at 50 μg/ml. For *Pseudomonads* Km, rifampin (Rif), and gentamicin (Gm) at 50, 100, and 50 μg/ml, respectively. Cycloheximide (100 μg/ml) was used to inhibit fungal growth in the rhizosphere competition assay.

Phylogenetic Analysis. The predicted AA sequences of the tail tube, sheath, and baseplate spike encoded within each tailocin region of *P. chlororaphis* 30-84 were used to identify related gene clusters in *P. chlororaphis*, *P. fluorescens,* and *P. protegens* strains using BLAST (Basic

Table 2.1. Bacterial strains and plasmids used in Chapter II.

Strain	Description/ Source	Reference
P. chlororaphis 30-84	Wild type, Rif^R	(72)
P. chlororaphis 30-84 ΔHolin	*hol* gene replaced with Km^R cassette; Km^R	(73)
P. chlororaphis 30-84 ΔTF1	*TF1* gene replaced with Km^R cassette	(76)
P. chlororaphis 30-84 ΔTF2	*TF2* gene replaced with Tc^R cassette	(76)
P. chlororaphis 30-84 ΔTF1/2	*TF1* and *TF2* gene replaced with Km^R and Tc^R, respectively,	(76)
P. chlororaphis 30-84 ΔBP1	*BP1* genes replaced with Km^R cassette,	(76)
P. chlororaphis 30-84 ΔBP2	*BP2* gene replaced with Tc^R cassette,	(76)
P. chlororaphis 30-84 ΔBP1/2	*BP1* and *BP2* gene replaced with Km^R and Tc^R, respectively,	(76)
P. chlororaphis 30-84 Δ*hol*	*hol* gene replaced with Km^R	(76)
Escherichia coli DH5α	F- *recA1endA1hsdRl7supE44thi-JgyrA96reLA1A (argF-lacZYA) 1169+801acZAM15/-*	GIBCO-BRL
Escherichia coli HB101	*F- hsdS20 (rB-mB-) supE44 recAl aral4proA2 lacYl galK2 rpsL20 xyl-5mtl-5I-*; GIBCO-BRL	GIBCO-BRL
P. putida F1	Rhizosphere Associated	ATCC
P. putida KT2440	Rhizosphere Associated	Dr. Gross (TAMU)
P. putida A514	Rhizosphere Associated	Dr. Gross (TAMU)
P. syringae pv. *tomato*	Plant Pathogen	(81)
P. syringae pv. phaseolicola	Plant Pathogen	Teaching collection
P. marginalis L	Plant Pathogen	Teaching collection
P. syringae pv. *syringae* B728A	Plant Pathogen	(82)
P. syringae pv. *tomato* DC3000	Plant Pathogen	(83)
P. syringae pv. *maculicola*	Plant Pathogen	Libo Shan Lab
P. aeruginosa PAK	Opportunistic Human Pathogen	(84)
P. aureofaciens 13485	Rhizosphere Associated	ATCC
P. fluorescens BL915	Rhizosphere Associated	(85)
P. syringae pv. *tabaci*	Plant Pathogen	Teaching collection

Table 2.1 Continued.

Strain	Description/ Source	Reference
P. protegens Pf-5	Rhizosphere Associated	(78)
P. fluorescens 2-79	Rhizosphere Associated	(75)
P. aeruginosa PAO1	Opportunistic Human Pathogen	(86)
P. fluorescens Q287	Rhizosphere Associated	(87)
P. fluorescens F113	Rhizosphere Associated	(88)
P. tolassi	Fungal Pathogen	(89)
P. aureofaciens Z1B	Rhizosphere Associated	Pierson Lab
Bacillus megaterium	Rhizosphere Associated	Teaching collection
Bacillus subtilis 613R	Rhizosphere Associated	Teaching collection
Pectobacterium carotovorum ATCC15713	Plant Pathogen	ATCC
Agrobacterium tumefaciens	Plant Pathogen	Teaching collection
Erwinia amylovora	Plant Pathogen	(90)

Local Alignment Search Tool). Homologous bacteriophage proteins were identified using the same approach. Sequence alignments and phylogenetic analyses were performed with MEGA7 (91). Amino acid alignments were performed with MUSCLE (MEGA7) and used to build maximum-likelihood trees using the Jones, Taylor, and Thorton (JTT) substitution model. The resulting phylogenies were edited with FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

Ultraviolet induction of *P. chlororaphis* 30-84 cultures. Ultraviolet (UV) irradiation was used to induce R-tailocin synthesis and cell lysis. Overnight LB cultures of *P. chlororaphis* 30-84 and the appropriate mutants were collected by centrifugation, washed with sterile water, resuspended in 20 mL of fresh LB (OD_{620}= 0.05), and grown at 28°C with shaking (200 rpm). When the cultures reached OD_{620}= 0.5, cultures were

centrifuged (7,500 x g) for 10 min. at 4°C. The pellets were washed once and resuspended in 10 ml of 0.85% NaCl. The 10 ml suspensions in sterile Petri plates were UV irradiated (400 μw/cm^2/sec) for 7 sec. with constant shaking of the plates to ensure even exposure to UV. The irradiated suspensions were transferred to foil covered 500 ml flasks containing LB (final concentration 1X, 50 ml). These cultures were grown at 28°C with shaking (200 rpm) and the optical density (620 nm) was monitored hourly. After cell lysis, the lysates were collected and filter sterilized to remove cellular debris.

Transmission Electron Microscopy. Transmission Electron Microscopy (TEM) was performed at the Microscopy and Imaging Center at Texas A&M University. UV-induced lysates were ultracentrifuged (2.5 hours at 48,000 x g) and resuspended in 2.2 mL of λ buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 8 mM $MgSO_4$) to generate a 40x concentration for TEM analysis. Tailocins were collected by dipping carbon-coated mica into the R-tailocin sample for one minute and staining with 2% aqueous uranyl acetate for 10 sec. The floating carbon film was attached to the grid and excessive liquid was removed for analysis. Specimens were visualized on a JEOL 1200EX TEM operating at an acceleration voltage of 100 kV. Images were recorded at calibrated magnifications by CCD camera, and measurements were acquired using Image J (92).

Soft-agar overlay spot assay. Killing activity of UV lysates was gauged using a soft-agar overlay assay similar to previously reported (93). Overnight cultures of *P. putida* KT2440 (and the strains listed in Table 2.1) were diluted to OD_{620}= 0.05 and grown at 28°C with shaking (200 rpm) until the culture reached OD_{620}= 0.4-0.5. Liquid LB containing 0.7% agar was kept at 55°C and 4 ml was transferred to 5-ml plastic

culture tubes (Falcon). Samples (100 µl) of the bacterial culture were added to the 4 ml liquid LB top agar, vortexed, and poured onto an LB agar (1.5%) plate. Plates were allowed to solidify for 10 min. before lysates were spotted. Lysates concentrated by ultracentrifugation and suspended in 5 ml of λ buffer were serially diluted and a concentration gradient of lysates was spotted (10 µl) on the plates in duplicate. Zones of growth inhibition without plaques, indicating the killing agent is non-replicative, signified tailocin activity.

Heterologous expression of *hol* and *endo* genes from *P. chlororaphis* 30-84. For gene expression studies in *P. chlororaphis* 30-84, a modified version of the expression vector pHERD20T (containing an arabinose-inducible promoter) was constructed by replacing the Ap^r cassette with the Km^r cassette of pUC4K (Table 2.2). The Km^r cassette was amplified using the primer set km-F/km-R (Table 2.3) and cloned into pHERD20T to create pHERD20TKm (Table 2.2). Primer pairs, hol-F/hol-R and endo-F/endo-R (Table 2.3), were used to amplify DNA fragments containing the native ribosome-binding site and the complete coding sequence of the holin and endolysin genes from *P. chlororaphis* 30-84, respectively. The endolysin PCR fragment was cloned together with the holin fragment or separately into the vector, pHERD20TKm generating a plasmid expressing both *hol* and *endo* (pHERD20TKmHE) and a plasmid expressing only *endo* (pHERD20TKmE) (Table 2.2). Plasmids were introduced by triparental conjugation.

Table 2.2. Plasmids used in chapter II

Plasmids	Characteristics	Source
pEX18Ap	Ap^R	(94)
pUC4K	Km^R, Ap^R, aph	(95)
pKRP12	Tc^R	(96)
pUCP20Gm	Gm^R	(97)
pHERD20TKm	pHERD20T derivative bearing Km^R cartridge; Km^R	(76)
pHERD20TKmHE	pHERD20TKm containing 946 bp *hol*::*endo* fragments; Km^R	(76)
pHERD20TKmE	pHERD20TKm containing 581 bp *endo* fragment; Km^R	(76)
pTF1	pUCP20Gm containing 3092bp Tail Fiber 1 and Chaperone fragment; Gm^R	(76)
pTF2	pUCP20Gm containing 4141bp Tail Fiber 2 and Chaperone fragment; Gm^R	(76)
pBP1	pUCP20Gm containing 2040bp fragment containing baseplate 1 assembly genes; Gm^R	(76)
pBP2	pUCP20Gm containing 2050bp fragment containing baseplate 2 assembly genes; Gm^R	(76)

Table 2.3. Primers used in chapter II.

Name	Sequence (5'-3')
TF1KO-UP-F	CGGAATTCGATGTGCGGCCCGAAACC
TF1KO-UP-R	CCAGAAGTTGGGATCCGTTGGTGAGCAGGGTGTAAT
TF1KO-DWN-F	GCTCACCAACGGATCCCAACTTCTGGCCTTGGCGGG
TF1KO-DWN-R	CCCAAGCTTGGGTGGTGAAGCTGTTGATGC
TF1 Comp-F	CGCGGATCCCGGCAGCCACGTCATCGGCG
TF1 Comp-R	CCCAAGCTTGTCGGTGCGGGGCGTTTATTGGA
TF1Check1-R	CCACAGCGAATCGCCATTGG
TF1Check3.1-F	GGGATTGCCCCTGGGACTTAC
TF1Check3.1-R	GGTTCCCATCAGAGTGGC
TF1Check2-F	GGGATTCAGTCTGCTGCCG
TF2KO-UP-F	CGGAATTCGTCACTCCCGGCCAAGTGATC
TF2KO-UP-R	CAACCGGCGGATCCGGCACGCTCTTTGGATAATCCATTGC
TF2KO-DWN-F	GAGCGTGCCGGATCCGCCGGTTGAAACTGATCTGAGACG
TF2KO-DWN-R	CCCAAGCTTCTTGCAGCTGTTGCTCCAGG
TF2 Comp-F	CCGGAATTCGCGTTTCGGCTCCAGTGCTC
TF2 Comp-R	CCCAAGCTTGGAGTTGTCCCTCGGTCAC
TF2check1-F	CTGAACGTCATCAAATCGGCCGG
TF2check1-R	GGGGTGTAGCCGAGCTTAAG
TF2check2-F	CTTCGGATGGCAATGACTGGG
TF2check2-R	CGGCCATCTACCAAGCCAAC
TF2check3-F	GCAAGCCCGATGCTTTCAG
TF2check3-R	CTGCGCCTCCTTCGTTGG
TF2-1-R	CCCAAGCTTGTCCTCGAACGCAAACG
TF2-2-F	CCGGAATTCCTGAACCCGCTTTGCGAACG
BP1-UP-F	CGGAATTCCCAGGCAGACGGCAACTTTG
BP1-UP-R	CACCTCGACGGTACCGCTCGTCAAACATGGCTCAGCCC
BP1-DWN-F	TTGACGAGCGGTACCGTCGAGGTGATGACGATATGACCG
BP1-DWN-R	CCCAAGCTTGAACTCCTTGCCCGATCCC
BP1-Comp-F	CGCGGATCCCGACCCCGACCAATACC
BP1-Comp-R	CCCAAGCTTGCGTCGAACTGTTGGGCGG
Base1KO1-F	GCGATCCGCTTCTTCAACCC
Base1KO1-R	CACCAGGCCAGCATCTGCTTG
Base1KO2-F	GCAGAAGCGCCCGTTGTATGG
Base1KO2-R	CCGGCCCCGAATTGATCTTG
BP2-UP-F	CGGAATTCAGTGCCAGGTGCGCATCG
BP2-UP-R	CTTACTCCAGCCGGATCCGGCGTGTCAGTAGGCTCATGG

Table 2.3 Continued.

Name	Sequence (5'-3')
BP2-DWN-F	CTGACACGCCGGATCCGGCTGGAGTAAGAACCGCCG
BP2-DWN-R	CCCAAGCTTCTGCTGGACACGAACCTCCAC
BP2-Comp-F	CCGGAATTCAGCTTGCTCGCGATGGGG
BP2-Comp-R	CCCAAGCTTCCTCCTCAAGTTCCGGCACC
Base2KO1-F	CGGCGAAGTGAACGACGACATGG
Base2KO1-R	ATGCGTTCCAGGGTCGACTCGTC
Base2KO2-F	GGTGCTGATCAAGCGCACCC
Base2KO2-R	GAGCCGGTGATCGTTCTCGC
Hol-UP-F	GGAATTCCTCGACGGTTCAGAGGGTTG
Hol-UP-R	GGCGTCCGAGGTACCGTTTGTCATGTCACTCCTCC
Hol-DWN-F	ATGACAAACGGTACCTCGGACGCCTGAACAACCGCC
Hol-DWN-R	CCCAAGCTTGTCAGCGCCTTGACCGATG
Hol check-1	CTCGACGGTTCAGAGGGTTG
Hol check-2	CCGGCTTTGTAGAGCTC
Hol check-3	GGAATTCCCAGGAGGAGTGACATGACAAAC
Hol check-4	GCGTATCGATCTGGACC
Hol check-5	CCCAAGCTTTTGTTCAGGCGTCCGAGCG
Hol-Comp-F	GGAATTCAGCCCATAACGCCAAAG
Hol-Comp-R	CCCAAGCTTTTGTTCAGGCGTCCG

[a] Underlined nucleotides are restriction sites added and the restriction enzymes are indicated at the end of primers.

Lysis cassette gene expression assays. To test the functionality of the lysis cassette, *P. chlororaphis* 30-84 strains containing pHERD20TKmHE or the control plasmid, pHERD20TKm, were grown overnight in AB + CAA supplemented with the appropriate antibiotics. These overnight cultures were diluted to OD_{620} ~0.1 and grown at 28°C with shaking. When the cultures reached OD_{620} ~0.2, half of the cultures were induced by the addition of arabinose (final concentration 1%). After induction, the optical density (620 nm) of the cultures was measured every hour to monitor cell lysis.

To examine the role of the endolysin in cell lysis, *P. chlororaphis* 30-84 strains containing pHERD20TKmE or the control plasmid, pHERD20TKm were grown and treated as in the previous experiment. However after arabinose induction, when the cell cultures reached OD_{620} ~1, chloroform was added to half of the cultures of cells (final concentration of 1% v/v), to permeabilize the cytoplasmic membrane enabling the endolysin to reach the peptidoglycan layer. The optical density of the cultures was measure every half hour after chloroform addition to monitor cell lysis.

Deletion of tail fiber, baseplate, and holin genes. *P. chlororaphis* 30-84 tail fiber mutants (ΔTF, that can no longer target susceptible strains) were generated using the suicide vector pEX18Ap (Table 2.2) as described by Hoang et. al. (94). To generate ΔTF1, sequences (1000 nt) flanking the tail fiber gene of tailocin 1 (pchl3084_1202) were amplified by two-step PCR using the primer pairs TF1KO-UP-F, TF1KO-UP-R and TF1KO-DWN-F, TF1KO-DWN-R, respectively (Table 2.3). Using the primer pair TF1KO-UP-F and TF1KO-DWN-R with the product of the previous PCR resulted in a construct that contained the upstream fragment separated from the downstream fragment by a *Bam*HI restriction site. This fragment was ligated into the *Eco*RI to *Hin*dIII site in the pEX18Ap multiple cloning region (94). The kanamycin resistance cassette with its promoter was then digested from a pUC4K (Table 2.2) template with the *Bam*HI enzyme and ligated between the upstream and downstream fragments of the tail fiber genes in pEX18Ap. The final construct was electroporated into *P. chlororaphis* 30-84 and transformants were plated onto LB amended with Km as performed previously. Double crossover mutants were obtained by counter-selection with LB amended with Km and

6% sucrose and confirmed using PCR primers specific to the internal regions of the tail fiber coding sequence (Table 2.3). Similarly, to generate ΔTF2, the sequences (1500 nt) flanking both tail fiber genes of tailocin 2 (pchl3084_1227-1230) were amplified by two-step PCR using the primer pairs TF2KO-UP-F, TF2KO-UP-R and TF2KO-DWN-F, TF2KO-DWN-R, respectively, followed by amplification using the primer pair TF2KO-UP-F and TF2KO-DWN-R (Table 2.3). This fragment was ligated into pEX18Ap at the *Eco*RI to *Hin*dIII site (94). The tetracycline resistance cassette with its promoter was digested from pKRP13 template with the *Bam*HI enzyme and ligated between the upstream and downstream fragments of the tail fiber genes in pEX18Ap. The final construct was electroporated into *P. chlororaphis* 30-84 and mutants selected using the appropriate antibiotics, as above. A double mutant ΔTF1/ ΔTF2 was generated by creating a tail fiber 2 deletion in a tail fiber 1 mutant (ΔTF1) and confirmed with the primers used to confirm tail fiber 1 and 2 mutants (Table 2.3). *P. chlororaphis* 30-84 base plate mutants (ΔBP, that no longer produce a tailocin particle) were generated as above. The ΔBP1 was generated by replacement of three genes, Pchl3084_1198-1120, with using a kanamycin antibiotic resistance marker and the primer set BP1-UP-F, BP1-UP-R, BP1-DWN-F, BP1-DWN-R (Table 2.3). Similarly, ΔBP2 was generated by replacement of three genes, Pchl3084_1221-1123, with a tetracycline antibiotic resistance marker using the primer set BP2-UP-F, BP2-UP-R, BP2-DWN-F, BP2-DWN-R (Table 2.2). All mutants were confirmed using PCR primers specific to the internal regions of the target gene coding sequence (Table 2.3).

Complementation of tail fiber, baseplate, and holin mutants. The deletion of the tail fiber coding sequences removed the ribosome-binding site of the genes downstream of both tail fiber deletions (Pchl3084_1203 and Pchl3084_1227-1230). Both of these genes (Pchl3084_1204 and Pchl3084_1231) are predicted to encode a chaperone that is required for the attachment of tail fibers to the tailocin baseplate. To ensure proper expression of both of these genes in the mutant background, the coding sequence of the tail fibers and their chaperones were PCR amplified with the primers TF1Comp-F and TF1Comp-R and TF2Comp-F and TF2Comp-R (Table 2.3). The resulting fragments were digested with the appropriate restriction enzymes and cloned into the expression vector pUCP20Gm (97). A similar method was used to complement the ΔBP1 mutant using the primers BP1-Comp-F and BP1-Comp-R (Table 2.3). The final constructions were introduced into the respective tail fiber or baseplate mutants by electroporation.

Biofilm replacement series analysis. A replacement series analysis was used to examine whether R-tailocin production confers a competitive advantage to *P. chlororaphis* 30-84 in mixed biofilms with *P. putida* KT2440 (98, 99). The replacement series consisted of different ratios of the two target strains in the starting inoculum, with the final cell density of 10^7 CFU/ml for all treatments. Treatments consisted of 100% *P. chlororaphis* 30-84 (wild type, ΔTF1, ΔTF1 pTF1, ΔTF2, ΔTF2 pTF2, ΔTF1/2,), 100% *P. putida* KT2440 or a 50:50 mixture of *P. chlororaphis* 30-84 strains and *P. putida* KT2440. The replacement series was used to infer competition by comparing the observed population sizes of each strain in mixed populations to the predicted sizes

based on the carrying capacity observed for the single strain treatments (e.g., rather than from the relative proportion of each strain in the final mixture). Total population density and ratios within the inoculum were confirmed by dilution plating. Biofilm cultures were grown as described previously (100). Briefly, bacterial strains were grown overnight in AB + CAA at 28°C with agitation (200 rpm), harvested, washed, and resuspended in fresh medium before creating the single strain or mixed starting cultures. Replacement series treatments (3 ml) were added to 15-ml polypropylene tubes, and the tubes were incubated at 28°C without shaking. After 48 h, the liquid and loosely adherent cells were removed by pipetting. The surface attached biofilm was washed three times with sterile water. After washing, 5 ml of phosphate buffered saline (pH 7.4) was added and the tubes were sonicated (3x, 10 sec) and vortexed (3x, 10 sec) to remove surfaced-adhered bacteria. Population sizes were quantified by dilution plating. Serial dilutions were plated on LB agar where *P. chlororaphis* 30-84 colonies were easily differentiated from *P. putida* KT2440 colonies without antibiotic selection by phenazine pigment production and differences in colony morphology. In this experiment, all strains carried a plasmid: either one or both of the tail fiber complements or the empty vector control pUCP20GM. Since no antibiotics were used in the competition assay, to confirm the stability of the plasmids in the complemented strains, dilutions were plated on media with and without gentamycin. No significant differences in CFU were found, indicating the plasmid was stable over the 48 hr assay (data not shown).

Rhizosphere replacement series analysis. A smaller replacement series analysis was conducted to examine the role of R-tailocins in *P. chlororaphis* 30-84 (wild type vs

ΔTF1/2) competition with *P. putida* KT2440 in mixed rhizosphere populations. This replacement series consisted of the same population ratios as described for the biofilm replacement series, but the total cell density of the treatments used to inoculate wheat seeds was 10^9 CFU/ml. The inoculum for the different treatments was prepared as above and final cell densities and ratios were confirmed via serial dilution plating. Wheat seeds (TAM304) were surface disinfested by incubation in 70% ethanol for 10 min followed by incubation in 90% commercial bleach (1 min) and then washed with sterile water (5x, 1min). Disinfested seeds were pregerminated on germination paper for 48 h and then the seedlings were suspended in the bacterial inoculum for 10 min. The inoculated seedlings were sown in autoclaved (45 min. twice with a 24 h break) wheat field soil (Bushland, Texas) and grown for 30 days (dark (8 hr) and light (16 hr) cycle at 27 ± 2°C). The rhizosphere bacterial populations were estimated as described previously (98). Briefly, the whole root system of each plant was collected, placed in 5 ml of phosphate buffered saline (pH 7.4), and sonicated (10 sec) and vortexed (10 sec. pulses) three times. Serial dilutions were plated on LB agar supplemented with cycloheximide and colonies were differentiated as above (e.g. without antibiotic selection). The roots were dried for 48 h in a 65°C oven and populations were standardized to dry root weight.

Statistical analysis

Comparisons of observed and expected surface attached biofilm populations were analyzed statistically using a student's t-test. *P* values less than 0.05 were considered significant.

Results

***P. chlororaphis* 30-84 genome encodes two R-tailocin particles.** The tailocin gene cluster of *P. chlororaphis* 30-84, situated between *mutS* (Pchl3084_1192) and the *cinA* (Pchl3084_1233) genes in the bacterial chromosome, is approximately 34 kb in length and consists of 40 putative ORFs (Fig. 2.1). The cluster encodes genes annotated as functioning in regulation, cell lysis and tailocin release, and tailocin particle assembly as well as cargo genes encoding proteins potentially carried with the tailocins (14, 32). At the beginning of the cluster, adjacent to *mutS*, is a gene with significant nucleotide sequence similarity to *ptrR,* the negative regulator of tailocin gene transcription in *P. aeruginosa* (14, 32). The cluster also contains a lysis cassette that consists of genes encoding a holin, an endolysin, and spanin complex involved in cell lysis and tailocin release. The genes encoding the lysis cassette flank the region containing the structural genes, with the holin upstream and the endolysin and spanin complex genes downstream of the structural genes. The region encoding the tailocin structural genes is nearly twice the size and has twice the number of ORFs as the genomic regions containing the R-tailocin structural genes previously described in other plant-associated pseudomonads (12, 14, 15, 19). This region can be divided into two smaller regions, each encoding the genes required for the assembly of different R-tailocins (Fig. 2.1). The order of the genes within each of the two R-tailocin assembly regions is different as are the sequences of

genes encoding proteins with similar functions. For example, the tail fiber and chaperone of the first tailocin are encoded by one gene each, whereas several genes encode the tail fibers and chaperones in the second cluster. The large cluster also includes two genes we refer to as cargo genes, Pchl3084_1195 and Pchl3084_1196 that are located immediately downstream of the holin and annotated as amidase domain-containing protein and the Putidacin L1 bacteriocin, respectively (14, 15, 32).

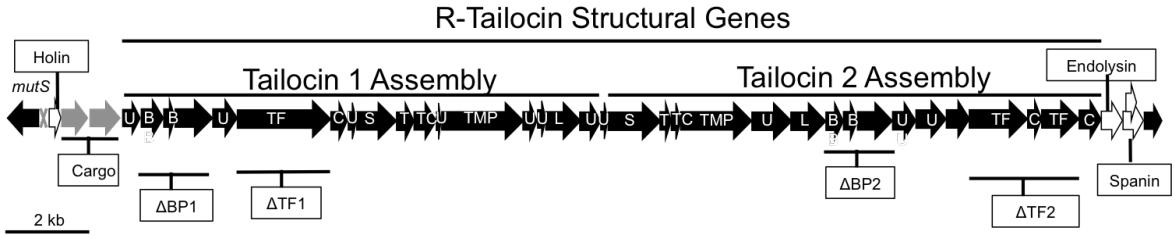


Figure 2.1. *P. chlororaphis* 30-84 R-Tailocin Gene Cluster. R-tailocin gene cluster is situated between the *mutS* and *cinA,* genes (Flanking, Black) within the chromosome of *P. chlororaphis* 30-84. This gene cluster contains 40 ORFS including a transcriptional regulator (gray X), a lysis cassette (white), cargo genes (gray), and R-tailocin structural genes (center, black). The lysis cassette is composed of the holin protein and endolysin enzyme (encoded by *hol* and *endo,* respectively) and the spanin complex encoded by *rz* and *rz1* (within the open reading frame of *rz*) and flanks the putative tailocin structural genes. The region encoding the R-tailocin structural genes is twice as large and contains twice as many genes as those observed in previously described clusters. This region can be divided into two sub-regions that encode all of the genes necessary to assemble two R-tailocin particles. The location of deletion mutations of the two tail fiber (ΔTF1 and ΔTF2) and the baseplate mutant (ΔBP1) are shown. Key: U, unknown; B, baseplate assembly; TF, tail fiber; C, tail fiber chaperone; S, sheath; T, Tail Tube; Tail Assembly Chaperone; TMP, Tape Measure Protein; L, Late Control D Protein.

R-tailocin gene clusters in *P. chlororaphis* 30-84 are evolutionarily distinct.

Examination of the tailocin gene clusters from the published genomes of other rhizosphere pseudomonads including strains of *P. chlororaphis*, *P. fluorescens,* and *P. protegens* revealed that several of these strains also had tailocin gene clusters predicted to encode more than one R-tailocin (Fig. 2.2). The tailocin gene clusters of all *P. chlororaphis* strains examined possess the genes predicted to produce two R-tailocins, with the exception of *P. chlororaphis* UBF2 that only encodes one. In contrast, the gene clusters of a majority of *P. fluorescens* strains inspected encode just one R-tailocin, with the exception of *P. fluorescens* SF4c and *P. fluorescens* Pf0-1, which encode two. Phylogenetic trees were built from multiple amino acid sequence alignments of the predicted proteins encoding structural components, including the tail tube, sheath, and baseplate spike (Fig 2.2). Interestingly, for the strains that appear to encode two R-tailocins, components of one tailocin always cluster together and components of the other always cluster together, suggesting that if the genome encodes two tailocins it is always the same two types. For the *P. chlororaphis* strains having two tailocins, the tailocin region designated tailocin 1 (Fig. 2.2) clusters with the *P. fluorescens* strains A506, 2-79, SBW25, and Q287, each encoding one tailocin (Fig 2.2). The R-tailocin 1 region sequences are most similar to the predicted amino acid sequences of *Vibrio parahaemolyticus* phage VP882 and *Halomonas aquamarina* phage phiHAP-1 (Fig. 2.2). Alternatively, the R-tailocin 2 region sequences cluster with the single tailocin sequences in *P. chlororaphis* UBF2, *P. protegens* Pf-5, and *P. fluorescens* Q8r1-96, as well as phyllosphere pathogens *P. syringae* pv. *syringae* B728a and *P. syringae* pv.

tomato DC3000. These are most similar bacteriophage SfV amino acid sequences (Fig. 2.2).

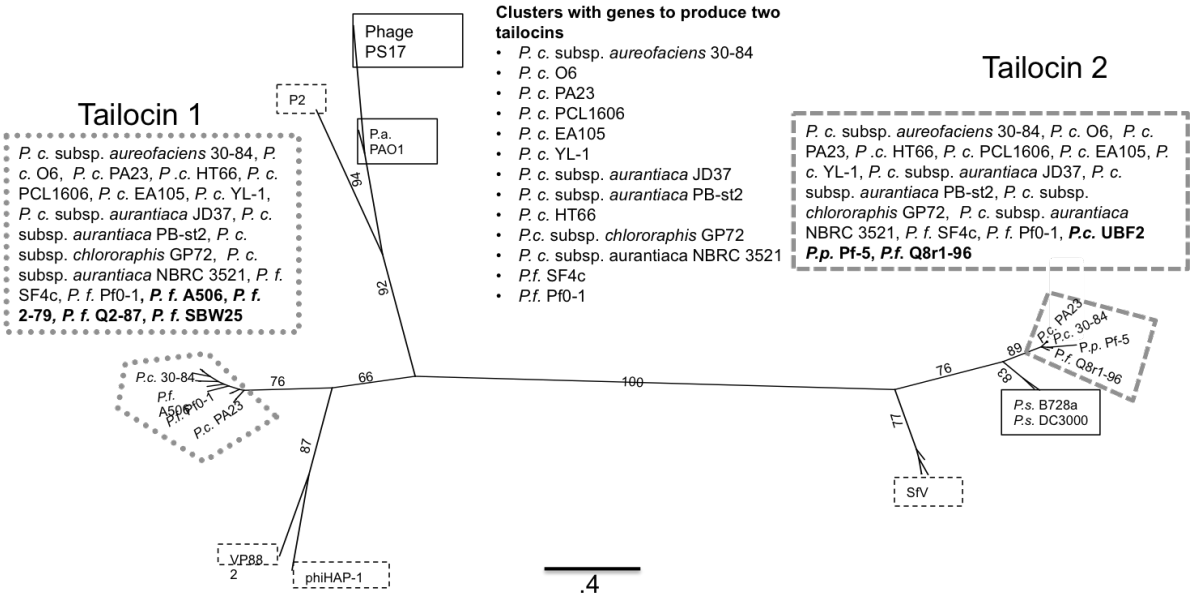
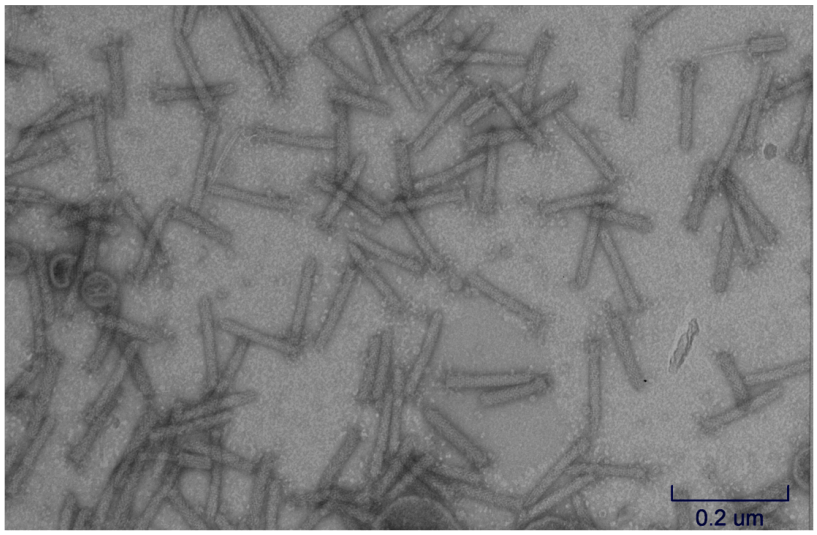


Figure 2.2. Phylogenetic analysis of tail tube proteins. The maximum likelihood tree was constructed from a multiple sequence alignment (MUSCLE) of the amino acid sequence of tail tube protein homologs from *Pseudomonas* R-tailocin clusters and bacteriophage genomes (Black dashed boxes). Two clusters containing the *P. chlororaphis* 30-84 tailocins are highlighted to denote the similarity of each R-tailocin (1 & 2) to other *P. chlororaphis* and *P. fluorescens* strains. The tail tube sequences from the R-tailocin gene region designated tailocin 1 (Circle, dashed gray boxes) cluster together and with some strains encoding also encoding two R-tailocin particles (plain font) as well as some encoding only one R-tailocin particle (bolded font). The R-tailocin 1 tail tube sequences are most closely related to the bacteriophages VP882 and phiHAP-1 (black, dashed box). The tail tube sequences from the region designated tailocin 2 (Rectangle dashed gray boxes) cluster together and with strains encoding two or one R-tailocin particle (plain vs bolded font, respectively) and are most closely related to bacteriophage SfV (black, dashed box). *P. aeruginosa* PAO1 and a few phyllosphere-colonizing *P. syringae* strains with tailocin clusters are shown for comparison (solid black box). Only select strain names are shown, but all of the strains used are listed in the boxes associated with the tree. The scale bar represents the number of AA substitutions per site. Bootstrap values (percent of 1,000 replications) are at branches. Abbreviations: *P. c. = P. chlororaphis*, *P. f. = P. fluorescens*, and *P. s. = P. syringae.*

***P. chlororaphis* 30-84 R-tailocin particles differ in length.** To characterize tailocin production in *P. chlororaphis* 30-84, an exponentially growing culture was exposed to UV light and the lysates were filter sterilized, concentrated by ultracentrifugation, and analyzed using transmission electron microscopy (TEM). The TEM micrograph revealed two distinct populations of rigid, rod-shaped, and capsid-less bacteriophage tail-like particles that differed in length (Fig. 2.3AB). Measurements of the lengths of R-tailocin particles using Image J confirmed that *P. chlororaphis* 30-84 produces two tailocin particles averaging 150 nm and 116 nm in length, respectively (Fig. 2.3B and 2.4A-C). The R-tailocin 1 cluster contains a larger (+ 97 AA) tape measure protein than the R-tailocin 2 cluster, which suggested that the R-tailocin 1 cluster encodes the larger R-tailocin particle. Consistent with this hypothesis, the smaller tailocin (118 nm ± 0.62) and larger tailocin (149 nm ± 0.19) were the only tailocins observed in the UV lysates of mutants in which baseplate genes of cluster 1 (ΔBP1) and cluster 2 (ΔBP2), respectively, were deleted (Fig. 2.1 and Fig. 2.4CD).

A



B

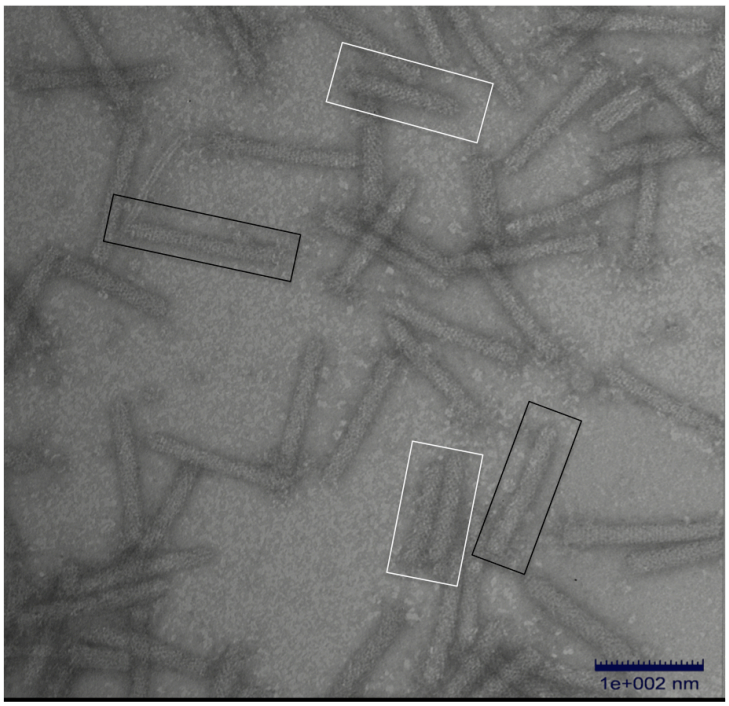


Figure 2.3. *P. chlororaphis* 30-84 may produce two distinct of R-tailocin particles. (A-B) Electron micrograph of *P. chlororaphis* 30-84 tailocins following UV induction. (B) Observation of UV-induced lysates using transmission electron microscopy revealed two distinct rigid, contractile bacteriophage tail-resembling particles that differ in length (white vs. black boxes). Samples were stained with 2% uranyl acetate.

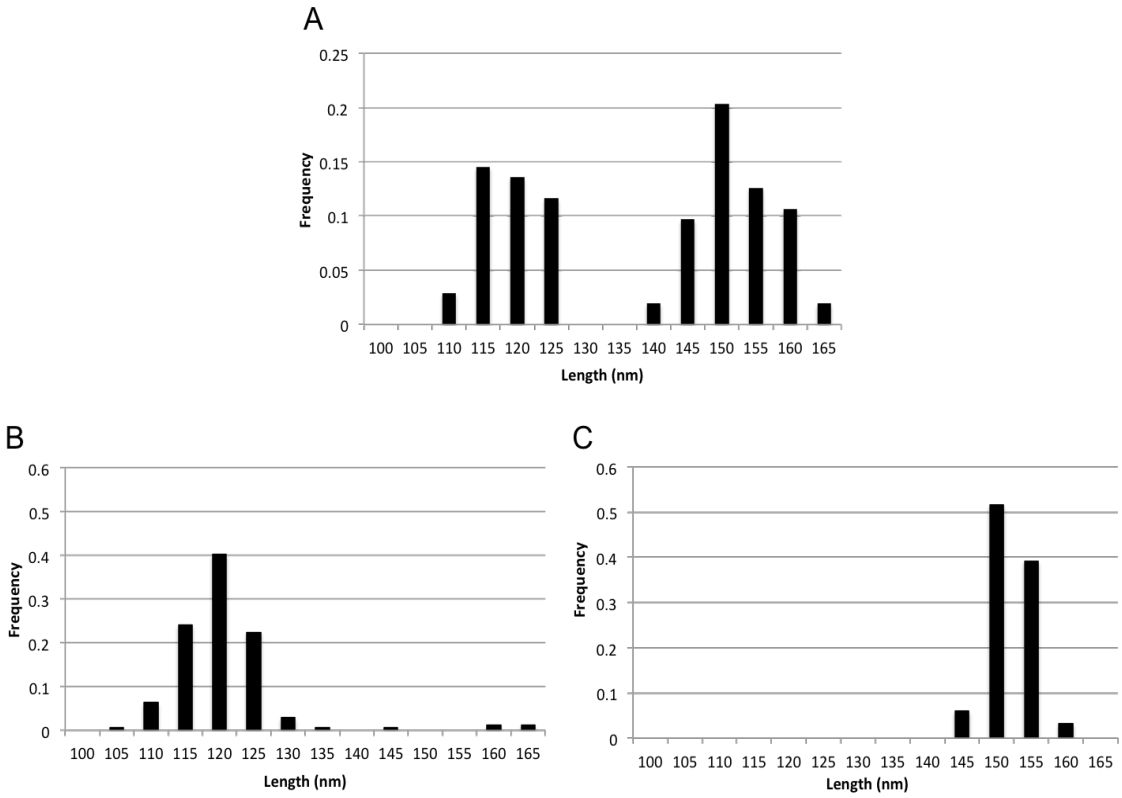


Figure 2.4. *P. chlororaphis* 30-84 produces two distinct populations of R-tailocin particles. (A) Frequency of tailocins in WT lysates measured in 5 nm categories. Measurement of the R-tailocin particles demonstrates that R-tailocin differ in length, 149nm $\pm$ 0.73 and 116nm $\pm$ 0.74. (B) Frequency of tailocins in ΔBP1 baseplate mutant lysate measured in 5 nm categories. Measurement of the R-tailocin particles demonstrates that the short (118 $\pm$ 0.62) R-tailocin is the only tailocin observed in the ΔBP1 UV lysates. (C) Frequency of tailocins in ΔBP2 baseplate mutant lysate measured in 5 nm categories. Measurement of the R-tailocin particles with image J demonstrates that the short (149 $\pm$ 0.19) R-tailocin is the only tailocin observed in the ΔBP2 UV lysates, indicating the tailocin 2 region encodes the larger tailocin.

Killing Spectrum of R-Tailocin Particles. Tail fiber mutants of the R-tailocin 1 (ΔTF1) or R-tailocin 2 particles (ΔTF2) or both R-tailocins were used to examine the killing spectrum of each *P. chlororaphis* 30-84 R-tailocin. In this experiment, the killing spectra of the mutants were compared to mutants complemented with a copy of the wild type gene *in trans*. UV- induced lysates were collected from the mutants and complements, serially diluted, and spotted onto soft agar overlays seeded with target bacterial species/strains. Tailocins were considered the killing agent when clearing zones without the formation of plaques were observed (Fig 2.5).

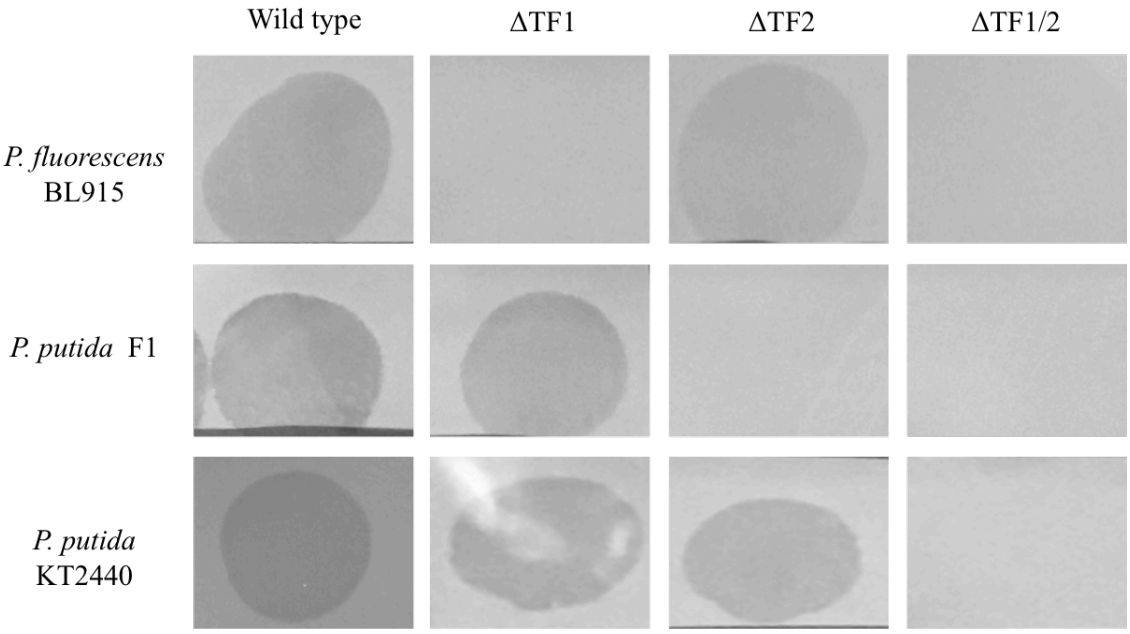


Figure 2.5. Soft agar overlay killing activity of *P. chlororaphis* 30-84 R-tailocins. Lysates were tested for killing activity as indicated by clearing zones when applied to soft agar lawns of the susceptible strains *P. fluorescens* BL915, *P. putida* FI, and *P. putida* KT2440. Five µl of filter sterilized, concentrated UV-induced lysates was spotted onto soft agar overlays seeded with the strains indicated (rows). The lysates were collected from wild type (*P. chlororaphis* 30-84), ΔTF1 (tailocin 1 tail fiber mutant), ΔTF2 (tailocin 2 tail fiber mutant), and ΔTF1/2 (double tail fiber mutant).

Among the strains tested, the host range of the tailocin particles was limited to *Pseudomonas* species (Table 2.4). The lysate of ΔTF1 lost the ability to kill *P. marginalis* and *P. fluorescens* BL915 (Fig. 2.5, Table 2.4). The lysate of the tail fiber 1 mutant (ΔTF1) lost the ability to kill *P. marginalis* and *P. fluorescens* BL915. Complementation of the ΔTF1 mutant with a plasmid borne copy of the gene and its chaperone restored the killing phenotype to the lysates, indicating that R-tailocin 1 targets *P. marginalis* and *P. fluorescens* BL915 (Table 2.4). The tail fiber 2 mutant (ΔTF2) lysate lost the ability to kill *P. putida* F1, *P. syringae* pv. *tomato* DC3000, *P. syringae* pv. *phaseolicola, P. syringae* pv. *tomato* A, *P. aeruginosa* PAK, and *P. aureofaciens* ATCC 13485. The ability to kill these strains was restored by complementation of ΔTF2, which demonstrates that R-tailocin 2 targets these strains (Table 2.4). Two strains, *P. putida* KT2440 and *P. syringae* pv. *syringae* B728a, were killed by both single-deletion mutants, but not the double tail fiber mutant (ΔTF1/2), which suggests that these strains possess the receptor targeted by both tailocin particles (Fig. 2.5). In fact, none of the strains tested were killed by the lysates collected from the double mutant, indicating that the tailocin particles are required for the killing activity (Table 2.4).

Table 2.4. *P. chlororaphis* 30-84 R-Tailocin Killing Spectrum.

Strain Tested	WT	ΔTF1	ΔTF1 pTF1	ΔTF2	ΔTF2 pTF2	ΔTF1/2
P. putida F1	+	+	+	-	+	-
P. putida KT2440	+	+	+	+	+	-
P. syringae pv. *syringae* B728a	+	+	+	+	+	-
P. syringae pv. *tomato* DC3000	+	+	+	-	+	-
P. syringae pv. *phaseolicola*	+	+	+	-	+	-
P. syringae pv. *tomato A*	+	+	+	-	+	-
P. marginalis	+	-	+	+	+	-
P. fluorescens BL915	+	-	+	+	+	-
P. aureofaciens ATCC 13485	+	+	+	-	+	-
P. aeruginosa PAK	+	+	+	-	+	-
P. fluorescens I-12	+	+	+	-	+	-
P. syringae pv. *maculicola*	+	+	+	-	+	-
P. putida A514	-	-	-	-	-	-
P. syringae pv. *tabaci*	-	-	-	-	-	-
P. protegens Pf-5	-	-	-	-	-	-
P. fluorescens 2-79	-	-	-	-	-	-
P. aeruginosa PAO1	-	-	-	-	-	-
P. fluorescens Q287	-	-	-	-	-	-
P. fluorescens F113	-	-	-	-	-	-
P. tolassi	-	-	-	-	-	-
P. aureofaciens Z1B	-	-	-	-	-	-

Table 2.4 Continued.

E. coli DH5α	-	-	-	-	-	-
Pectobacterium carotovorum ATCC 15713	-	-	-	-	-	-
Bacillus subtilis 613R	-	-	-	-	-	-
Bacillus megaterium	-	-	-	-	-	-
Agrobacterium tumefaciens	-	-	-	-	-	-

*"-" Indicates no killing activity and "+" indicates killing activity.

***P. chlororaphis* 30-84 *hol* and *endo* are involved in R-tailocin release.** The *hol* and *endo* genes of the lysis cassette in the tailocin gene cluster were characterized in order to confirm their role in R-tailocin release. The two genes were cloned either together or separately downstream of an arabinose inducible promoter in the vector, pHERD20T. Co-expression of the *hol* and *endo* genes in *P. chlororaphis* 30-84 resulted in cell lysis (Fig. 2.6A), whereas the empty vector controls and the non-induced cells did not lyse under these conditions. In contrast, expression of the *endo* gene alone did not alter growth following induction (Fig. 2.6B), indicating that without the activity of the holin protein, the endolysin is unable to reach and degrade the peptidoglycan layer. However, when cells expressing *endo* were treated with chloroform (1 %), the cells rapidly lysed compared to cells expressing *endo* that were not treated with chloroform (Fig 2.6B). Treatment of the wild type (having the empty vector) with chloroform also resulted in cell lysis, but to a much lesser extent and at a slower rate, indicating the

endolysin was important for rapid cell lysis. In this experiment, the chloroform substituted for the holin by permeabilizing the cytoplasmic membrane so that the endolysin could reach the peptidoglycan layer. These experiments were repeated in *E. coli* and produced similar results (data not shown), indicating the functionality of these proteins is not limited to *Pseudomonas*.

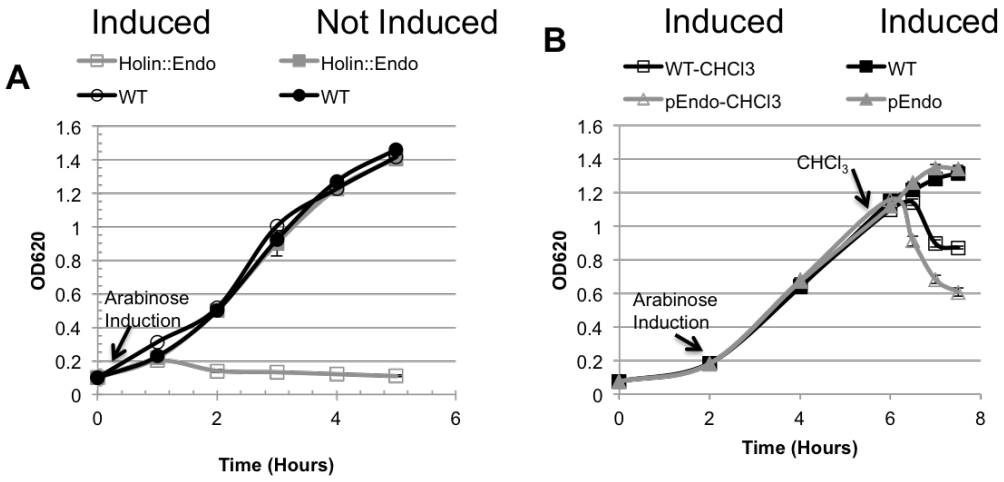


Figure 2.6. Role of *hol* and *endo* in R-Tailocin-associated cell lysis. (A) Wild type cells containing either the Hol::Endo plasmid or the empty vector were grown in AB + CAA and cell density measured OD_{620}. Cultures were either induced or not induced with arabinose. Expression of the *hol::endo* construct resulted in cell lysis following arabinose induction (Open Square), whereas non-induced cultures (Closed Square) continued to grow at the same rate as wild type containing the empty vector (Open Circles: induced, Closed circles: non-induced). (B) Arabinose induced expression of *endo* without the *hol* gene did not result in altered growth patterns compared to wild type with the empty vector (Closed Triangle versus Closed Square). Whereas, permeabilization of the cytoplasmic membrane with chloroform (1% $CHCl_3$) resulted in rapid cell lysis (Open Triangle). Treatment of wild type (having the empty vector) with chloroform also resulted in cell lysis, but to a lesser extent and at a slower rate (Open Square).

A previously characterized *hol* mutant (73) was used to evaluate the role of holin in R-tailocin release. Consistent with the described role of holins in bacteriophage release, the *P. chlororaphis* 30-84 holin deletion mutant does not lyse and continues to elongate hours after UV irradiation, whereas the wild type cell lysed 2 hours after induction (Fig. 2.7) (101). Induced cultures of the holin mutant were monitored for an additional 5 hours and the cells still had not lysed. At 7 hours post UV induction, 1% chloroform was added to the culture and the cells lysed rapidly, which yielded functional R-tailocins (data not shown). These observations indicated that UV-induced holin mutants do not lyse but endolysin and R-tailocin particles accumulate in the cytoplasm. These data confirm that the holin is an important component of the lysis cassette required for cell lysis and release of both R-tailocins produced by *P. chlororaphis* 30-84.

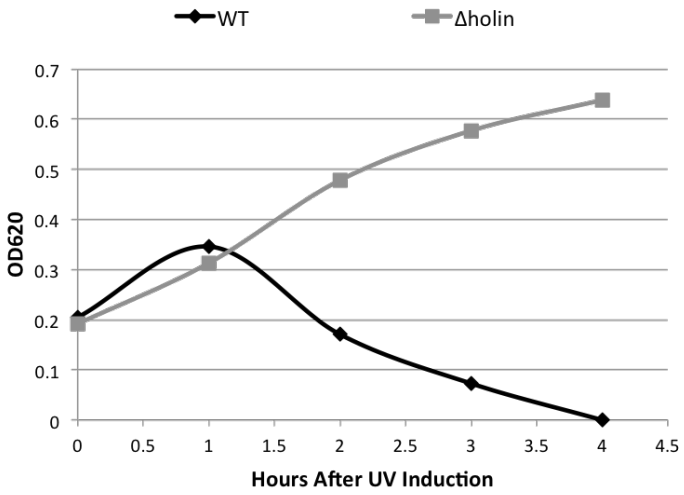


Figure 2.7. The role of *hol* in R-tailocin associated cell lysis. The holin mutant does not lyse after UV induction (Grey Square), whereas the wild type does (Black Diamond), indicating that the holin is required for cell lysis and release of the R-tailocin particles. Data are the means from nine biological replicates from three independent experiments.

R-tailocin production bestows a competitive advantage in surface attached biofilms. To determine whether R-tailocins produced by *P. chlororaphis* 30-84 contribute to the competitive fitness of *P. chlororaphis* 30-84 within mixed-species biofilms, a replacement series competition analysis was performed using wild type or tail fiber mutant derivatives of *P. chlororaphis* 30-84 and *P. putida* KT2440 (sensitive to both R-tailocins). The replacement series consisted of a *P. chlororaphis* 30-84 strain and *P. putida* inoculated in different ratios (e.g. ratios 1:0, 0.5:0.5, 0:1) maintaining a constant final cell density. Populations recovered from biofilms inoculated with either strain alone indicated the carrying capacity for each of the strains under these conditions. The carrying capacity of the *P. chlororaphis* 30-84 wild type and tail fiber mutant

populations were significantly greater than that of *P. putida* KT2440, but not significantly different from each other (Fig. 2.8A-F). The similarity in the ability of the wild type and tail fiber mutants to establish biofilm populations suggest that mutation to R-tailocin tail fibers does not influence the development of surface attached biofilms under these conditions. Comparison of the final densities of each strain in the mixed population to their carrying capacities controls for differences between strains in carrying capacity and indicates their competitive fitness. Expectations are that in the absence of competition, each strain would attain a population size equivalent to 50% of its carrying capacity (since there was 50% less of it in the initial mixed inoculum, compared to the single strain inoculum). However, in the surface attached biofilms wild type *P. chlororaphis* 30-84 populations are significantly larger, whereas *P. putida* KT2440 populations are significantly smaller than would be predicted in the absence of competition, indicating that the wild type has a competitive advantage over *P. putida* KT2440 in mixed surface attached biofilms (Fig. 2.8A). Similarly, single tail fiber mutants ΔTF1, ΔTF2, and their complements competitively excluded *P. putida* KT2440 in surface attached biofilm populations (Fig. 2.8B-E). In contrast, the populations of the double mutant (ΔTF1/2) and *P. putida* KT2440 did not differ from the expected population sizes when mixed in a surface attached biofilm (Fig. 2.8F), indicating loss of both tailocins results in the loss of the *P. chlororaphis* 30-84 competitive advantage over *P. putida* KT2440. Taken together, these data demonstrate that the production of at least one functional R-tailocin is required for *P. chlororaphis* 30-84 to have a competitive advantage over *P. putida* KT2440 in surface attached biofilms

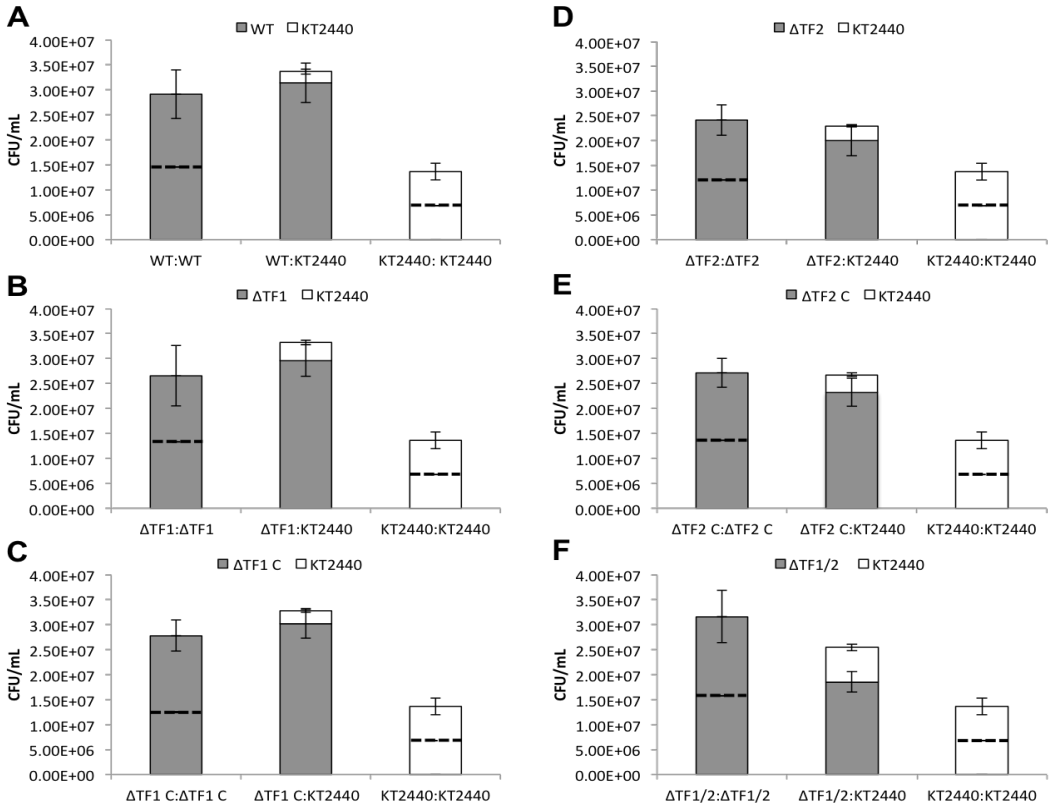


Figure 2.8. Role of R-tailocin in Biofilm Competition. Wild type *P. chlororaphis* 30-84 (WT) and R-tailocin sensitive *P. putida* KT2440 (KT2440) or one of the *P. chlororaphis* 30-84 tail fiber mutants (ΔTF) and KT2440 were grown in biofilm conditions in AB minimal medium using a replacement series design (e.g. strains were introduced separately or in a 50:50 mixture, all treatments had the same total cell density 10^7 CFU/ml). Surface attached biofilm populations were harvested after 48 hours and quantified by dilution plating. Final population sizes of single strain treatments indicate the carrying capacity of each strain under the experimental conditions. Expectations are that in the absence of competition, each strain would attain a population size equivalent to 50% of its carrying capacity and this 50% expected population size is indicated by a line across the bars of each single strain treatment. (A) *WT vs. KT2440*: In mixed surface attached biofilm communities, WT populations are significantly larger, whereas KT2440 populations are significantly smaller than expected, indicating that WT populations outcompeted KT2440. (B-E) Tail Fiber mutants vs. KT2440: In mixed biofilms, ΔTF1, ΔTF1C, ΔTF2, ΔTF2C populations are larger, whereas KT2440 populations are smaller than expected, indicating that these populations outcompeted KT2440. (F) In mixed surface attached biofilm communities, ΔTF1/ΔTF2 and KT2440 population sizes are no different than the expected population sizes, indicating that the competitive advantage is lost with disruption of the tail fiber genes within both R-tailocin clusters. Data points represent the means from fifteen biological replicates from four independent experiments and error bars indicate standard errors.

R-tailocin production accords a competitive advantage in the rhizosphere.

To determine whether the competitive advantage of R-tailocin production observed in mixed biofilms translates into a competitive advantage in the wheat rhizosphere, the replacement series experiment was repeated on wheat roots. The carrying capacity of wild type *P. chlororaphis* 30-84 and ΔTF1/2 populations in the wheat rhizosphere were not significantly different (Fig 2.9AB). This indicates that tailocin activity does not influence the ability of *P. chlororaphis* 30-84 to form rhizosphere populations in soil with reduced native microbial populations. In mixed populations, wild type *P. chlororaphis* 30-84 populations were larger than predicted whereas *P. putida* KT2440 populations were smaller than predicted, indicating that wild type *P. chlororaphis* 30-84 was competitively excluding *P. putida* KT2440 in mixed species rhizosphere communities (Fig. 2.9A). In mixed rhizosphere communities, the *P. chlororaphis* 30-84 double tail fiber mutant and *P. putida* KT2440 population sizes were similar to the expected population sizes, indicating that the benefit to *P. chlororaphis* 30-84 is diminished with the loss of tailocin activity (Fig. 2.9B).

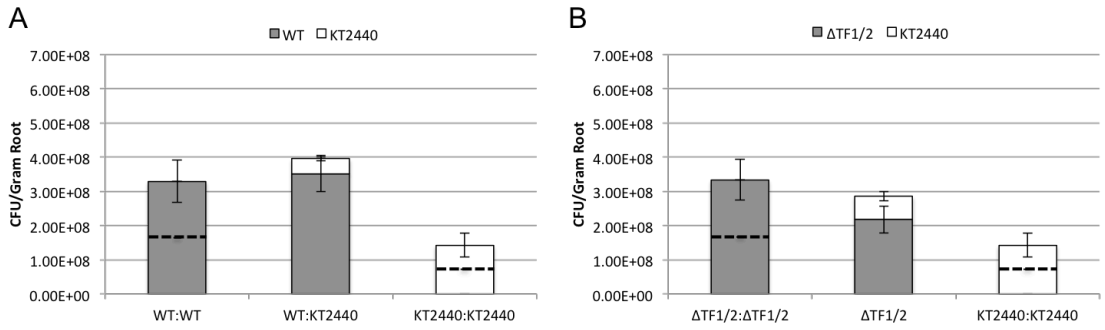


Figure 2.9. Role of R-tailocin in Rhizosphere Competition. Wheat seeds (TAM304) were surfaced sterilized, pre-germinated and dip-inoculated with each treatment (final concentration10^9 CFU/ml). After inoculation, seeds were planted in autoclaved wheat field soil and rhizosphere populations were harvested 30 days after planting and quantified by dilution plating. (A) In mixed rhizosphere communities, WT populations reach the same final population size as wild type inoculated alone and the KT2440 populations are smaller than the expected population size, suggesting WT benefits at the expense of KT2440 in mixed rhizosphere populations. (B) In mixed rhizosphere communities, the double tail fiber mutant and KT2440 population sizes were similar to the expected population sizes, indicating that the benefit to WT is lost with loss of tailocin activity. Similar to the biofilm, there was no difference in the ability WT and the double tail fiber mutant to survive on plant roots in autoclaved soil. Data points represent the means from six biological replicates from one experiment and error bars symbolize standard errors.

Discussion

The *P. chlororaphis* 30-84 R-tailocin gene cluster contains twice as many structural genes as similar clusters in other plant-associated *Pseudomonas* species, such as *P. fluorescens* A506, *P. putida* BW11M1 (14, 32), *P. protegens* Pf-5 (78), and *P. syringae* pv. *syringae* B728a (15). Bioinformatic analysis revealed the region potentially encodes the structural components to produce two R-tailocins that share a single lysis

cassette. The two R-tailocin structural regions have distinct ancestries based the maximum likelihood phylogenies showing that R-tailocin 1 and 2 are most closely related to the phages VP882/phiHAP-1 and SfV, respectively. Moreover, we found that the majority of the *P. chlororaphis* strains examined also potentially encode two R-tailocins with high amino acid similarity to R-tailocin 1 and R-tailocin 2 produced by *P. chlororaphis* 30-84. The speculation that some *Pseudomonas* species, including *P. fluorescens* SF4c, may produce more than one R-tailocin was reported previously (33), however our study expands previous comparisons to include *P. chlororaphis* strains. Recent work categorized the R-tailocins produced by *Pseudomonas* species into four groups corresponding to evolutionary relationships and denoted by species complex of origin (14, 33). These groups include Rp1, Rp2, Rp3, and Rp4, represented by tailocins produced by *P. aeruginosa*, *P. putida*, *P. fluorescens*, and *P. syringae*, respectively. Using the classification system developed by Ghequire et al. (14), our results place the R-tailocin 1 of the *P. chlororaphis* strains in the Rp3 group and the R-tailocin 2 in the Rp4 group. Our study goes beyond bioinformatic characterization of the tailocin region by demonstrating that *P. chlororaphis* 30-84 produces two distinct, functional R-tailocin particles and suggests that pseudomonads encoding similar tailocin gene clusters also likely produce two different tailocins.

The R-tailocin gene cluster of *P. chlororaphis* 30-84 bears similarities to those of some plant-associated *Pseudomonas* species, but differs in many ways from the gene clusters identified in *P. aeruginosa* and *P. syringae* species. For example, the *P. chlororaphis* 30-84 R-tailocin gene cluster is integrated into the bacterial chromosome

between *mutS* and *cinA*, which is similar to the insertion point observed in some plant-associated *Pseudomonas,* yet is different from *P. aeruginosa* and *P. syringae* where the cluster is located between *trpE-G* (14, 15). The *P. chlororaphis* 30-84 R-tailocin lysis cassette encodes a holin, endolysin, and spanin module akin to other plant-associated pseudomonads ; this spanin module is often missing from *P. aeruginosa* tailocin gene clusters (14). The *P. chlororaphis* 30-84 cluster also contains genes referred to as cargo genes in other plant-associated pseudomonads (14, 15). One of these (Pchl3084_1195) is predicted to encode a N-acetylmuramoyl-L-alanine amidase, potentially involved in peptidoglycan biosynthesis and hydrolysis, and has 96-98% amino acid sequence similarity to cargo genes in tailocin gene clusters in other plant-associated *P. chlororaphis* strains (e.g. PA23, O6, 1606). The other (Pchl3084_1196) shares significant amino acid sequence similarity to the lectin-like bacteriocins LlpA1 (50 %) and LlpA2 (49 %) produced by *P. protegens* Pf-5 (102). Previous studies in *P. putida* suggested that the LlpA protein is physically associated with the R-tailocin particle produced by this strain (14). Further work is needed to determine whether there is a relationship between these proteins and R-tailocin activity.

The two *P. chlororaphis* 30-84 R-tailocins differ in size and killing spectrum. TEM micrographs of concentrated lysates revealed the presence two populations of rigid, contractile capsid-less bacteriophage-tail resembling particles that differ by length. Deletion of the genes required to assemble each of theR-tailocin baseplates confirmed that that R-tailocin regions 1 and 2 encode the larger and smaller particles, respectively.

Using specific tail fiber mutants, we demonstrated that the killing spectrum of both R-tailocins was limited to *Pseudomonas*, however, within *Pseudomonas* species the killing spectrum of the two tailocins differed. These findings suggest that the tailocins target different receptors. The finding that lysates collected from the double tail fiber mutants did not kill any of the strains screened indicated that the production of functional R-tailocin particles is required for killing activity. Our results demonstrate that the production of more than one functional R-tailocin particle increases the killing spectrum of the producing strain, thereby increasing strain competitiveness. Ongoing work seeks to identify the receptors targeted by each of the *P. chlororaphis* 30-84 R-tailocins.

R-tailocins confer a competitive advantage in biofilms and the rhizosphere. Tailocin production was required for competitive survival against an R-tailocin-sensitive strain in surface attached biofilm and rhizosphere populations. The use of a replacement series design was necessary to determine direct competition between strains, because *P. putida* KT2440 and *P. chlororaphis* 30-84 differed in their ability to form *in vitro* biofilms and rhizosphere populations. We observed that wild type populations had a strong competitive advantage over *P. putida* KT2440 in mixed biofilms and rhizosphere. However, loss of functional R-tailocin production resulted in the loss of the competitive advantage over *P. putida* KT2440 in mixed biofilms and the rhizosphere. The importance of R-tailocins as determinants of interactions among closely related strains of *P. aeruginosa* was shown previously (45, 51, 53). Although the killing capacities of R-tailocins produced by plant-associated *Pseudomonas* strains have been described, to our

knowledge this is the first demonstration that R-tailocins function in competition between plant-associated bacterial species in surface attached biofilms or in rhizosphere communities.

Recent conceptualizations of root tip colonization provide a framework for relating root development and bacterial population dynamics (56). As bacteria are recruited from the soil to the root tip region, attach and develop into microcolonies, the root environment changes as the root tip matures and develops. The simultaneous maturation of the many proliferating root tips creates a highly diverse environmental landscape composed of recruitment niches at the tips that evolve into mature root niches within a matter of days. The need for competitive strategies targeting closely related combatants likely changes as the root and root-inhabiting microbial populations develop. In future studies, regard for the dynamics of microbial populations in relation to root tip maturation may identify niches where R-tailocin production is most effective.

CHAPTER III

FUNCTION OF R-TAILOCIN PRODUCTION BY *P. CHLORORAPHIS* IN THE ECOLOGY OF SOIL-ROOT BACTERIAL COMMUNITIES

Introduction

Soil-rhizosphere microbial communities are among the most complex and diverse communities studied in terms of their composition, structure and function. Plants actively recruit a rhizosphere microbiome from bulk soil, a subset of which consists of plant growth-promoting rhizobacteria (PGPR) that facilitate nutrient uptake, protect against pathogens and abiotic stress, and contribute to plant growth and fitness (57, 60). PGPR must be able to persist in bulk soil among the native microbiota and compete with these microorganisms for relatively more abundant resources in the root rhizosphere.

Bacteria utilize interference strategies to outcompete their neighbors for nutrients within natural communities, such as the production of antibiotics, including bacteriocins. Bacteriocins are narrow spectrum antibacterial proteins produced by both Gram negative and positive bacteria (3, 4, 7, 32, 76). They are generally classified into two broad categories, low and high molecular weight (6, 8). Low molecular weight bacteriocins such as colicins and S-type pyocins, consist of two proteins: a larger protein that possesses the killing function and a smaller immunity protein that protects the producer

cell. High molecular weight bacteriocins are multi-subunit bacteriocins that resemble bacteriophage tails (8).

Phage tail-like bacteriocins, or tailocins, include non-contractile F-tailocins and contractile R-tailocins that resemble and are evolutionarily related to *Siphoviridae* and *Myoviridae* phage tails, respectively (9, 11-13). R-tailocin particles are a double hollow cylinder composed of a sheath and core connected to a baseplate with six tail fibers (9, 36, 37). Tailocin production is typically induced by the SOS response and the particles are released extracellularly by cell death and lysis (12, 34, 76-78). After release, R-tailocin tail fibers interact with specific cell surface receptors, commonly lipopolysaccharides or lipooligosaccharides of susceptible bacteria (10, 14, 31, 45, 46, 54). This interaction triggers irreversible contraction of the particle and insertion of the core through the cell wall of the susceptible cell, depolarizing the membrane, which results in the death of the targeted cell (38, 39, 41, 50). R-tailocins are extremely efficient killers, often a single particle is sufficient to kill a cell (44).

Experimental and genomic studies have shown that R-tailocins are produced by or their gene clusters are present in the genomes of a diversity of bacteria (13-16, 18-22, 24, 33, 50, 76), suggesting that they may be important for competitive interactions or other unknown aspects of bacterial fitness. However, the ecological role of R-tailocins in the natural environment of the producer has received little attention. Studies exploring their function typically focus only on pairwise interactions between an R-tailocin producer and R-tailocin sensitive strains. For example, tailocin production provided an advantage to the opportunistic human pathogen *P. aeruginosa* over specific susceptible

P. aeruginosa strains in pairwise interactions in mixed cultures (51, 52). Similarly, in pairwise interactions the production of xenorhabdicin, a R-tailocin particle produced by *Xenorhabdus* species, conferred an advantage over sensitive strains (26, 27).

Within the rhizosphere, bacteria typically exist in multispecies aggregates or biofilm communities wherein they must interact with different species competing for similar rhizosphere commodities (58, 103, 104). A better understanding of the mechanisms governing the establishment and persistence of PGPR within the native microflora community could facilitate the design of effective strategies to maximize beneficial PGPR-plant interactions to enhance plant health (63), including the potential deployment of bacteriocins to manipulate plant-associated bacterial communities and control plant pathogens populations (6, 105). *Pseudomonas chlororaphis* 30-84 is a good candidate for studying the ecological function of R-tailocins in the rhizosphere because R-tailocin production by this strain has been described previously (76). *P. chlororaphis* 30-84 is a phenazine-producing PGPR that was isolated from the rhizosphere of wheat for use in the management of wheat take-all disease (72). Comparison of available genomic sequences indicates that similar to other *P. chlororaphis* and some *P. fluorescens* strains, but different from other plant-associated *Pseudomonas* species (i.e., *P. putida*, *P. syringae*), the R-tailocin gene cluster of *P. chlororaphis* 30-84 encodes two distinct R-tailocins, both of which are released by a shared lysis cassette (15, 33). The two *P. chlororaphis* 30-84 R-tailocin particles have different killing spectra and potentially different ancestral origins (76). The production of R-tailocin particles provides *P. chlororaphis* 30-84 a competitive advantage in pairwise interactions with a

tailocin sensitive strain in mixed species biofilms *in vitro* and on wheat roots in autoclaved soil (76). However, the role of R-tailocin production in the survival and persistence of *P. chlororaphis* within the complex rhizosphere microbiome or bulk soil remains to be determined. Interestingly, the genetic module encoding the second R-tailocin in the *P. chlororaphis* 30-84 R-tailocin gene cluster (tailocin 2) contains two different genes annotated as tail fibers. Currently, it is unclear whether the genes encode different tail fibers, and if so whether they are incorporated onto the same tailocin particle producing a chimera with two different tail fibers, or incorporated onto different particles resulting in the production of two distinct tailocin 2 particles potentially having different target specificities. In either case, we hypothesize that the production of two different tail fibers enhances the killing spectrum of tailocin 2, and thereby the overall competitive capacity of *P. chlororaphis* strains in the rhizosphere microbiome.

The aims of this study were to (i) determine the functionality of the two tail fibers encoded by genes within the tailocin 2 genetic module, (ii) estimate the breadth of influence of tailocin production on the diversity of microbes found in the rhizosphere i.e., by screening a collection of culturable bacteria isolated from the wheat rhizosphere to determine the proportion that are targeted by each of the tailocins produced by *P. chlororaphis* 30-84, and (iii) examine the role of R-tailocin production in the persistence of *P. chlororaphis* 30-84 in competition with the community of organisms comprising natural microbiomes of the wheat root-rhizosphere and of bulk soil.

Experimental Procedures

Bacterial Strains and Media. The bacterial strains and plasmids used in this study are described in Table 3.1. The wheat rhizobacterial collection screened for tailocin susceptibility is similar to the one used previously to screen for isolates that positively or negatively influence *P. chlororaphis* 30-84 quorum sensing (106, 107). The collection is a random sample of culturable bacteria found on wheat roots and may include siblings of the same isolates. The bacteria were isolated from seven different fields in three separate geographic locations (South-central Arizona, USA; Ottawa, Canada; and Pullman, Washington, USA). A spontaneous rifampin-resistant derivative of *P. chlororaphis* 30-84 was used in all studies. *P. chlororaphis* 30-84 and wheat rhizosphere strains were grown at 28°C in Luria Bertani (LB) medium or King's medium B (106, 107). Antibiotics were used when appropriate at the following concentrations: kanamycin (km), rifampin (Rif), gentamycin (Gm) and tetracycline (Tc) at 50, 100, 50, and 50 μg/ml, respectively.

Table 3.1. Bacterial strains used in Chapter III.

Strain	Description/Source	Reference
P. chlororaphis 30-84	Wild Type, Rif^R	W.W. Bockus
P. chlororaphis 30-84 ΔBP1	replaced with Km^R cassete	(76)
P. chlororaphis 30-84 ΔBP2	replaced with Tc^R cassete	(76)
P. chlororaphis 30-84 ΔBP1/2	replaced with Km^R cassete, replaced with Tc^R cassete	(76)
P. chlororaphis 30-84 ΔTF2	*TF2* genes replaced with Km^R cassete	(76)
P. syringae pv. *phaseolicola* N4SP	Plant Pathogen	(76)
P. syringae pv. *tomato* DC3000	Plant Pathogen	(76)
P. putida F1	Rhizosphere Associated	(76)
P. aureofaciens ATCC13485	Rhizosphere Associated	ATCC
Xanthomonas campestris pv. *vesicatoria*	Plant Pathogen	Teaching Collection
Escherichia coli DH5α	F- recA1endA1hsdRl7supE44thi-JgyrA96reLA1A (argF-lacZYA) 1169+801acZAM15/-	GIBCO-BRL

Ultraviolet induction of *P. chlororaphis* 30-84 cultures. R-tailocin production was induced using a method similar to that described previously (10, 76) . Briefly, overnight LB cultures of *P. chlororaphis* 30-84 and the appropriate mutants were collected by centrifugation, washed with sterile water, resuspended in 20 mL of fresh LB (OD_{620}= 0.05), and grown at 28°C with shaking (200 rpm). When the cultures reached OD_{620}= 0.5, cultures were centrifuged (7,500 x g) for 10 min. at 4°C. The pellets were washed once and resuspended in 10 ml of 0.85% NaCl. The 10 ml suspensions in sterile Petri plates were irradiated with ultraviolet light (UV) (400 μw/cm^2/sec) for 7 sec with constant shaking of the plates to ensure even exposure to UV. The irradiated suspensions

were transferred to foil-covered 500 ml flasks containing LB (final concentration 1X, 50 ml). These cultures were grown at 28°C with shaking (200 rpm) and the optical density (620 nm) was monitored hourly. After cell lysis, the lysates were collected, centrifuged (7,500 x g), and filter sterilized to remove cellular debris. For use in soft-agar overlay experiments, the UV-induced lysates were concentrated by ultracentrifugation (2.5 hours at 48,000 x g). The pellet was suspended in 1 mL of λ buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 8 mM $MgSO_4$).

Tailocin purification. A method similar to that described in Yao *et al.* (10) was used for large scale preparation and purification of tailocin particles. Briefly, ammonium sulfate (final conc. 40% wt/vol) was slowly added to cell free, UV-induced tailocin lysate stirring at 4°C and incubated at 4°C for 18 hours. The ammonium sulfate precipitate was pelleted (17,000 x g for 1 hour at 4°C and resuspended in 1/10 of the original volume in cold Tn_{50} buffer (10mM Tris [pH 7.5], 50mM NaCl). This suspension was dialyzed overnight against Tn_{50} buffer with a Slide-A-Lyzer dialysis cassette (3.5 molecular weight cutoff). Tailocin particles were then sedimented at 90,619 x g for 2.5 hours at 4°C and the pellet were resuspended in λ buffer.

***In trans* complementation of tailocin 2 tail fiber genes.** In chapter II, two tail fiber genes were identified in the tailocin 2 gene cluster of *P. chlororaphis* 30-84 (76). These genes and their associated chaperone genes were cloned together or separately into the expression vector pUCP20Gm (Table 3.2) (97). The primers TF2-1-F and TF2-1-R were used to PCR amplify the first tail fiber and chaperone gene of tailocin 2, TF2-1. The second tail fiber and chaperon gene, TF2-2, was PCR amplified with the primer

set TF2-2-F and TF2-2-R (Table 3.2). The first and second tail fiber genes were PCR amplified together using the primer set, TF2-1-F and TF2-2-R. The DNA fragments resulting from these PCR reactions were digested with the appropriate restriction enzymes and cloned into pUCP20Gm. The final constructions were introduced into a mutant strain (ΔTF2) that had both tail fiber genes replaced with a Km^R cassette (76).

Table 3.2. Plasmids and Primers used in Chapter III.

Plasmid	**Description/Source**	**Reference**
pUCP20Gm	Gm^R, pUCP20 with *Sma*I-flanked Gm^R cassette inserted into unique *Sca*I site within *bla*	(76)
pGT2	Gm^R, GFP-based promoter trap vector containing a promoter less *gfp* gene	(108)
pBP1	pGT2 containing 2051bp fragment that includes the baseplate genes Pchl3084_1198, _1199, and _1200	This study
pBP2	pGT2 containing 2063bp fragment that includes the baseplate genes Pchl3084_1221, _1222, and _1223	This study
pTF2-12	pUCP20Gm containing 4141bp tail fiber 2 genes and there associated chaperone genes	(76)
pTF2-1	pUCP20Gm containing 2138bp tail fiber 2-1 gene and its associated chaperone gene	This study
pTF2-2	pUCP20Gm containing 2083bp tail fiber 2-2 gene and its associated chaperone gene	This study

Primers	
Name	**Sequence (5' to 3')***
BP1-Comp-F	CGCGGATCCCGACCCCGACCAATACC
BP1-Comp-R	CCCAAGCTTGCGTCGAACTGTTGGGCGG

Table 3.2 Continued.

Name	Sequence (5' to 3')*
BP2-Comp-F	CCGGAATTCAGCTTGCTCGCGATGGGG
BP2-Comp-R	CCCAAGCTTCCTCCTCAAGTTCCGGCACC
TF2-1-F	CCGGAATTCGCGTTTCGGCTCCAGTGCTC
TF2-1-R	CCCAAGCTTCCTTCTGCCTCGAATATGAC
TF2-2-F	CCGGAATTCCTGAACCCGCTTTGCGAACG
TF2-2-R	CCCAAGCTTGGAGTTGTCCCTCGGTCAC

Soft-agar overlays. Killing activity of the purified UV lysates was gauged using a soft-agar overlay assay similar to that previously described (93). Overnight cultures of bacteria were collected by centrifugation, washed with sterile water, and suspended to OD_{620}= 0.05. Liquid LB containing 0.7% agar was kept at 55°C and 4 ml was transferred to 5-ml plastic culture tubes (Falcon). Samples (100 µl) of the bacterial solution were added to the 4 ml liquid LB top agar, vortexed, and poured onto an LB agar (1.5%) plate. Plates were allowed to solidify for 10 min. before the purified lysates were spotted. Concentrated purified lysates were diluted in 5 ml of sterile water, serially diluted, and spotted (5 µl) on plates in duplicate. Overlay plates were incubated overnight at 28°C. Zones of growth inhibition without plaques, indicating the killing agent is non-replicative, signified tailocin activity. To characterize the two tail fiber genes in the tailocin 2 genetic module, tailocins were harvested from ΔTF2 strains expressing one or

both tail fiber genes *in trans*. These tailocins were spotted onto lawns of strains previously shown to be susceptible to tailocin 2 (Table 3.1). For the rhizosphere screen, tailocins were harvested from wild type, ΔBP1 (no tailocin 1), ΔBP1 (pBP1), ΔBP2 (no tailocin 2), ΔBP2 (pBP2), and ΔBP1/2 (doesn't produce either tailocin) and spotted onto lawns seeded with one bacterial strain from the wheat rhizosphere collection described above.

Preincubation / killing assay. To determine whether the two tail fibers are incorporated onto the same tailocin 2 particle a preincubation / killing assay was conducted (22). In this experiment, a tailocin solution is mixed with cells to promote the binding of tailocin particles to susceptible cells. The tailocin-cell mixture is then centrifuged to pellet the cell-bound tailocins and the supernatant (contains unattached tailocin particles) is collected. The killing activity of the unbound tailocins within the supernatant is quantified by spotting serial dilutions onto lawns seeded with susceptible cells. If the tail fibers incorporate onto separate particles, a reduction in killing activity would depend on the pre-incubation strain, e.g., pre-incubation with a strain targeted by TF2-1 would reduce killing activity against strains targeted by TF2-1, but not those targeted by TF2-2. For the assay, a purified tailocin 2 solution was incubated with solutions of resistant strains (*P. chlororaphis* 30-84), susceptible strains (*P. aureofaciens* ATCC13485, *P. syringae* pv. *tomato* DC3000, *P. syringae* pv. *phaseolicola*) (10^9 CFU/mL), or sterile water (control) for 30 min. at 4°C. After pre-incubation, the remaining tailocin killing activity was determined by spotting serial dilutions of cell free supernatants onto lawns of susceptible strains. Pre-incubation supernatants were

collected by centrifugation (21,000 X *g* for 10 min), filter sterilized, serially diluted, and spotted onto lawns of *P. aureofaciens* ATCC13485, *P. syringae* pv. *tomato* DC3000, or *P. syringae* pv. *phaseolicola*. Quantification of tailocin activity was expressed as arbitrary units (AU/mL), the reciprocal of the highest dilution factor that inhibited growth of the indicator strains (17, 109).

Rhizosphere persistence assay. A rhizosphere persistence assay was used to better understand the contribution of R-tailocin production to colonization and persistence in the wheat rhizosphere (75). Wild type *P. chlororaphis* 30-84 containing the vector alone (WT pGT2) or mutants deficient in the production of one or both tailocin (ΔBP1 pGT2, ΔBP1 pBP1, ΔBP2 pGT2, ΔBP2 pBP2, ΔBP1/2 pGT2) were inoculated into autoclaved (121^{o}C for 45 minutes 2X with a 24 h break) or natural wheat field soil (Uvalde, Texas), sown with disinfested wheat seedlings (TAM304), and grown for 20 days (8 h dark 16h light cycle) at 28°C. Bacteria were grown overnight with antibiotic selection, washed twice with sterile water, and resuspended in sterile water (20 mL) to a final concentration of 1x10^{9} CFU/mL. This suspension was mixed with soil to a final concentration of 10^{6} CFU/g by diluting the suspension ten-fold in 20 mL (sterile water) and then adding the diluted suspension to soil (500 g) and mixing thoroughly. Wheat seeds (TAM304) were surface disinfested by incubation in 70% ethanol for 10 min. followed by incubation in 10% bleach (10 min.) and washed with sterile water 3X. Disinfested seeds were pregerminated on seed paper for two days and the seedlings were sown four days after the bacteria were inoculated into the soil. There were 50 plants for each soil treatment. After 20 days of growth, 10 of the 50 plants from each soil treatment

were harvested and sampled for population sizes, as previously described (76). Briefly, the entire root system of each plant was collected, placed in 5 ml of phosphate buffered saline (pH 7.4), and sonicated (and vortexed (3X each) for 10 sec. Bacterial suspensions were diluted serially and plated onto LB supplemented with Rif and Rif + Gm. The roots were dried for 48 h at 65°C and populations were standardized to dry root weight. The shoots of the unharvested plants were removed at the soil surface and the soil and root system was poured into a 0.473-liter paper cup, mixed by shaking, and returned to the tube from which it was taken. This soil was incubated for 4 d at 15° C and then replanted with disinfested, pregerminated wheat seeds. The repeat harvest cycling process exposed the strains to progressively more intense competition from the indigenous rhizosphere-root community. The plasmid used for *in trans* complementation was stable in all derivatives of *P. chlororaphis* throughout the course of this experiment (data not shown).

Bulk soil persistence assay. The bulk soil populations of WT pGT2, ΔBP1 pGT2, ΔBP1 pBP1, ΔBP2 pGT2, ΔBP2 pBP2, and ΔBP1/2 pGT2 were quantified at every harvest interval. One gram bulk soil (i.e. the soil not associated with plant roots) samples were collected during every harvest cycle. Bacterial populations were determined by suspending the soil sample in 1 ml sterile water, sonicating and vortexing (2X each) for 10 sec. followed by serial dilution of the sample and plating onto medium amended with the appropriate antibiotics.

Statistical Analyses. Rhizosphere and soil persistence assays were analyzed statistically by comparing the means of treatments using Analysis of Variance ($P< 0.05$) and Fisher's protected least significant difference tests.

Results

Tailocin 2 has two functional tail fibers. The genetic module encoding tailocin 2 in the *P. chlororaphis* genome contains two genes annotated as tail fibers (i.e. TF2-1 and TF2-2). In a previous study, a mutant strain that had both tail fibers replaced by an antibiotic resistance cartridge**,** ΔTF2, was used to describe the killing spectrum of tailocin 2 (76). To elucidate the function of the tailocin 2 tail fiber genes, each tail fiber gene (and its chaperone) were cloned separately or together into the expression vector, pUCP20Gm and introduced into ΔTF2 (Table 3.2). The ΔTF2 mutant and the ΔTF2 mutant complemented for production of each tail fiber, e.g. ΔTF2 (pTF2-1) or ΔTF2 (pTF2-2), were UV-induced and tailocins were collected from lysates. Complementation of ΔTF2 with TF2-1 *in trans* restored killing activity against *P. putida* F1, *P. syringae* pv. *phaseolicola* N4SP, *P. syringae* pv *tomato* DC3000, and *Xanthomonas campestris* pv. *vesicatoria*, but did not restore killing activity against *P. aureofaciens* ATCC13485 (Fig. 3.1, Table 3.3). Complementation of ΔTF2 with TF2-2 restored killing activity against *P. aureofaciens* ATCC13485, but did not restore killing activity against any of

the other strains tested. Simultaneous expression of TF2-1 and TF2-2 restored killing activity against all strains tested (Fig. 3.1, Table 3.3). These results indicate that each tail fiber alone is sufficient for killing activity but confers a different killing spectrum to tailocin 2.

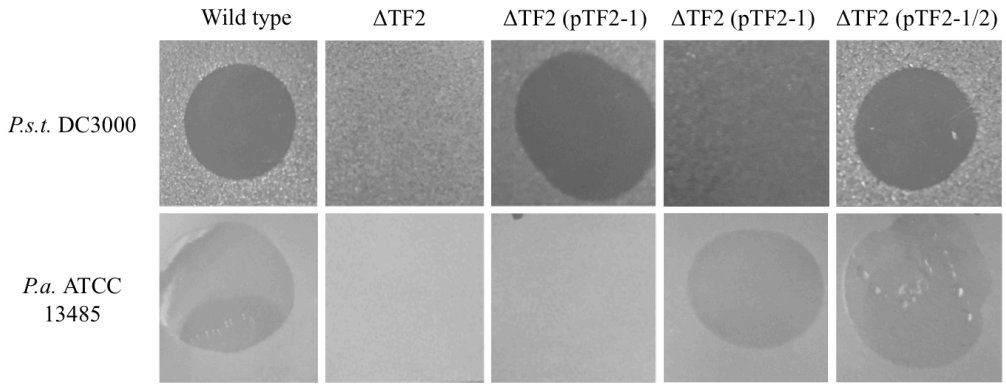


Figure 3.1. Killing activity of ΔTF2 complemented with each of tailocin 2 tail fibers. 5µl of purified tailocin solution was spotted onto soft agar overlays seeded with select strains (rows). Deletion of the both tailocin 2 tail fibers (ΔTF2) results in loss of killing activity against *P. syringae* DC3000 (*P.s.t.* DC3000) and *P. aureofaciens* 13485 (*P.a.* 13485). *In trans* expression of the first tail fiber, TF2-1, restored killing activity against *P.s.t.* DC3000. *In trans* complementation of the second tail fiber, TF2-2, returned killing activity against *P.a.* 13485. Simultaneous *in trans* expression of both tail fibers restored killing activity against all susceptible strains tested.

Table 3.3. Killing spectra of tailocin 2 tail fibers.

Strains	ΔTF2 (empty vector)	ΔTF2 (pTF2-1)	ΔTF2 (pTF2-2)	ΔTF2 (pTF2-12)
***P.a.* ATCC13485**	-	-	+	+
***P.p.* F1**	-	+	-	+
P.s. phaseolicola	-	+	-	+
***P.s.t.* DC3000**	-	+	-	+
X.c. vesicatoria	-	+	-	+

*"-" Indicates no killing activity and "+" indicates killing activity. Abbreviations: *P.a.*, *Pseudomonas aureofaciens*; *P.p., Pseudomonas putida*; *P.s.*, *Pseudomonas syringae*; *P.s.t.*, *Pseudomonas syringae* pv. *tomato*; *X.c., Xanthomonas campestris*.

The different tail fibers could be incorporated onto the same or separate tailocin particles. To address this question, the target specificity of a tailocin 2 solution was pre-incubated either with sterile water (control), a sensitive strain targeted by each tail fiber (*P. aureofaciens,* TF2-2; and *P. syringae,* TF2-1), or a strain resistant to both (*P. chlororaphis* 30-84). After pre-incubation, the remaining tailocin killing activity was determined by spotting serial dilutions of cell free supernatants onto lawns of susceptible strains. If the tail fibers were incorporated onto the same tailocin particle, a reduction in killing activity is expected regardless of the susceptible strain with which each is pre-incubated because binding to the receptor of a susceptible cell triggers the irreversible contraction and inactivation of particles. If the tail fibers incorporate onto separate particles, a reduction in killing activity would depend on the pre-incubation strain. Under this scenario, pre-incubation with a strain targeted by TF2-1 would reduce killing activity against strains targeted by TF2-1, but not those targeted by TF2-2 (i.e., only tailocins with TF2-1 would contract). Our data show that as expected incubation of tailocin 2 particles with tailocin 2 resistant cells (e.g., *P. chlororaphis* 30-84) did not

result in a reduction in killing activity against *P. aureofaciens* or *P. syringae* compared to the control (Table 3.4). Moreover, the killing activity was not reduced when the susceptibility of the preincubation and test strains differed, e.g., pre-incubation with *P. syringae* strains, target *P. aureofaciens* or pre-incubation with *P. aureofaciens* and target either *P. syringae* strain. Reduced killing activity was observed only when the pre-incubation and test strain were targeted by the same tail fiber e.g., *P. aureofaciens* vs. *P. aureofaciens* or *P. syringae* vs. *P. syringae* (Table 3.4). These results indicate that two different tailocin 2 particles are synthesized, one with TF2-1 and another with TF2-2, and confirm that they have different killing spectra.

Table 3.4. Tailocin killing activity after incubation with resistant or susceptible strains

	Killing Activity (AU/mL)		
Preincubation Strain	***P.a.* ATCC13485**	***P.s.t.* DC3000**	***P.s. phaseolicola***
Control	4.17E+04	1.04E+06	2.08E+05
***P.c.* 30-84**	4.17E+04	1.04E+06	2.08E+05
***P.a.* ATCC13485**	3.33E+02	1.04E+06	2.08E+05
***P.s.t.* DC3000**	4.17E+04	8.33E+03	3.33E+02
P.s. phaseolicola	4.17E+04	8.33E+03	8.33E+03

*Abbreviations: *P.a.*, *Pseudomonas aureofaciens*; *P.p., Pseudomonas putida*; *P.s.*, *Pseudomonas syringae*; *P.s.t.*, *Pseudomonas syringae* pv. *tomato*; *X.c., Xanthomonas campestris*. Experiment was repeated 5 times, and a representative data set is shown.

Wheat rhizobacteria are susceptible to *P. chlororaphis* 30-84 R-tailocins. In order to characterize the importance of R-tailocin production in bacterial interactions in the rhizosphere, a previously described collection of bacterial isolates from wheat roots grown in Arizona, Canada, and Washington State (106, 107) was screened for sensitivity to *P. chlororaphis* 30-84 tailocins. Tailocin sensitivity was indicated by the formation of a killing zone where the tailocin sample was spotted (Fig. 3.2). Approximately 12% (56/484) of all bacterial isolates screened were targeted by either tailocin 1 or tailocin 2. Of the targeted strains, nearly 11% (52/484) were targeted by tailocin 2, whereas only 0.6% (3/484) and 0.2% (1/484) were targeted by tailocin 1 or both tailocins, respectively (Fig. 3.2, Table 3.5). The proportions of susceptible strains were fairly consistent (e.g., less than 15%) among the areas where the bacteria were isolated: *P. chlororaphis* 30-84 tailocins targeted approximately 13% (49/363), 4% (2/46), and 7% (5/75) of the isolates collected from Washington State, Canada, and Arizona, respectively, and tailocin 2 typically targeted a greater percentage of the strains tested than tailocin 1 (Table 3.5). Approximately 27% (24/89) of the strains screened that were fluorescent on Kings Media B (typically indicative of fluorescent pseudomonads) were susceptible to one of the tailocins, suggesting that as expected, not all pseudomonads were susceptible to the tailocins produced by *P. chlororaphis* 30-84. Approximately 8% (32/395) of non-fluorescent strains were targeted, suggesting that some strains susceptible to these tailocins may not be pseudomonads.

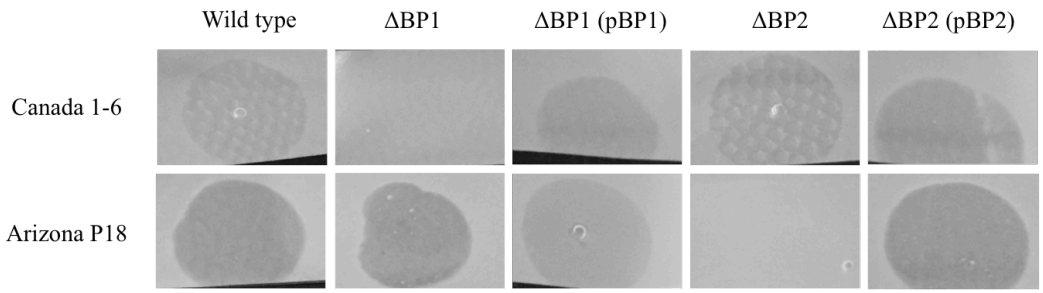


Figure 3.2. Killing activity of R-tailocins produced by *P. chlororaphis* 30-84 and several tailocin-deficient mutants against selected strains. 5µl of purified tailocin solution was spotted onto soft agar overlays seeded with select wheat rhizobacterial strains (rows). ΔBP1 tailocins (tailocin 1 deficient) did not form killing zones on the isolate Canada 1-6, and ΔBP2 tailocins (tailocin 2 deficient) did not form killing zones on isolate Arizona P18.killing to both *P.s.t.* DC3000 and *P.a.* 13485.

Table 3.5. R-tailocin susceptibility.

Location	Number of strains	Tailocin 1	Tailocin 2	Both	Total
Arizona	75	0%	7%	0%	7%
Canada	46	2%	2%	0%	4%
Washington State	363	0.6%	13%	0.3%	13%
Total	484	0.6%	11%	0.2%	12%

*% Indicates the percentage of the population susceptible

Tailocin production contributes to persistence in the wheat rhizosphere but not in bulk soil. A repeat harvest assay was employed to better understand the role of tailocin production in the colonization and persistence of *P. chlororaphis* in the wheat rhizosphere (75). Populations of *P. chlororaphis* 30-84 and the double tailocin mutant ΔBP1/2 enumerated from the roots (and closely adhering soil) of wheat plants grown in autoclaved soil (~10^8 CFU/Gram) were significantly greater than populations from

wheat grown in natural wheat field soil (~10^6 CFU/Gram), indicating that the presence of the native microbiota influences *P. chlororaphis* 30-84 population dynamics (Fig 3.3AB). In autoclaved soil, populations of WT and ΔBP1/2 were similar in size after three successive harvest cycles (Fig. 3.3A). However in natural field soil, although none of the populations differed at the first harvest, WT populations were larger than those of ΔBP1, ΔBP2, and ΔBP1/2 after the second and third harvest (Fig 3.3B). Complementation *in trans* of each tailocin mutant resulted in populations similar in size to WT after every harvest cycle (Fig. 3B). These results indicate that both R-tailocins produced by *P. chlororaphis* contributed to persistence in the wheat root rhizosphere under these experimental conditions.

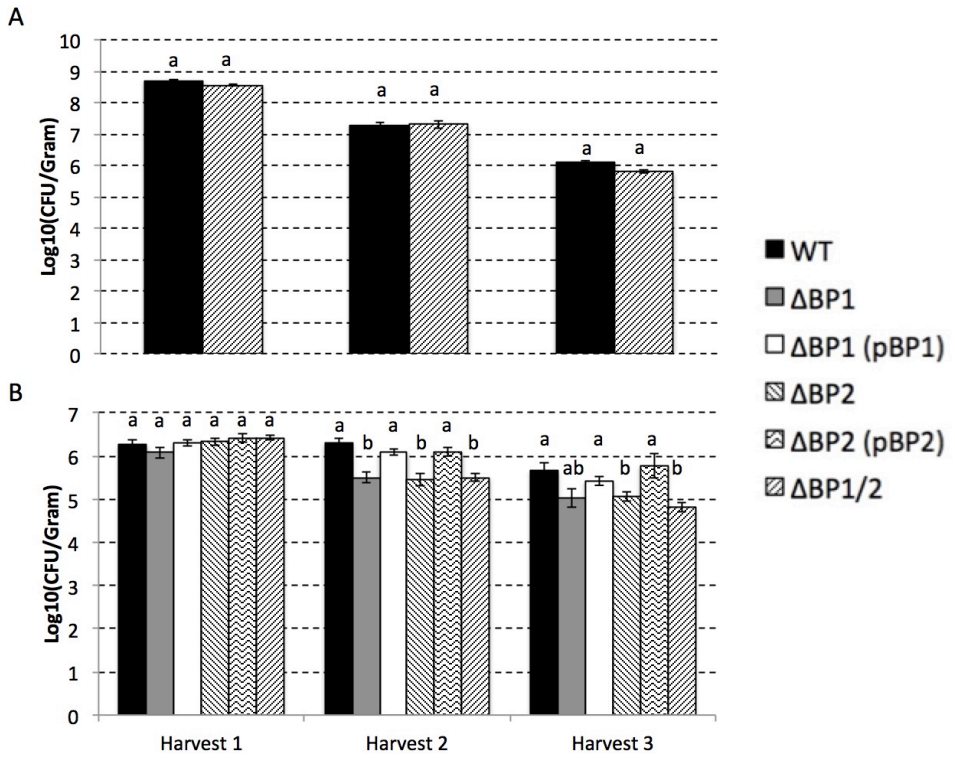


Figure 3.3. Bacterial populations in the wheat rhizosphere after repeated harvest cycles in autoclaved and natural soil. Inoculum of wild type and the tailocin baseplate mutants having the empty vector as a control (WT, ΔBP1, ΔBP2, ΔBP1/2) or having the vector carrying the complement of the mutation (ΔBP1 pBP1, ΔBP2 pBP2) inoculated into natural or autoclaved wheat field soil (Uvalde, TX). Surfaced sterilized, pregerminated wheat seeds (TAM304) were sown into the inoculated soil and grown 20 days. The rhizosphere populations were quantified by serial dilution plating (CFU/Gram of wheat root). The shoots of plants not harvested were removed, the root system and soil were mixed and incubated, and then sterilized, pregerminated wheat seeds were sown into the recycled soil. (A) Plants grown in autoclaved soil (B) or natural soil. Data points represent the means from 10 biological replicates and error bars indicate standard errors. The experiment was repeated twice with similar results. For each harvest cycle, values with different letters are significantly different.

Populations also were enumerated within the bulk soil (i.e., not adherent to the wheat roots) from the same containers used for the previous experiment. Bulk soil populations of wild type and ΔBP1/2 (~10^6 CFU/Gram) persisting in autoclaved soil

were significantly larger than populations persisting in natural wheat field soil (~10^5 CFU/Gram), but only after the first harvest cycle (Fig. 3.4AB), indicating that in bulk soil the natural microbiota also influences *P. chlororaphis* 30-84 bulk soil population dynamics, but to a lesser extent than in the rhizosphere. As in the rhizosphere assay, the bulk soil populations of WT and ΔBP1/2 were no different in autoclaved soil after three harvest cycles, demonstrating that tailocin production does not influence bulk soil persistence when native microbiota are reduced by autoclaving (Fig. 3.4A). In contrast to the rhizosphere assay, in natural wheat field soil the bulk soil populations of WT and tailocin mutants (ΔBP1, ΔBP2, and ΔBP1/2) were not significantly different after any of the harvest cycles (Fig. 3.4B), indicating that under these experimental conditions there is no measurable advantage of R-tailocin production for persistence in bulk soil.

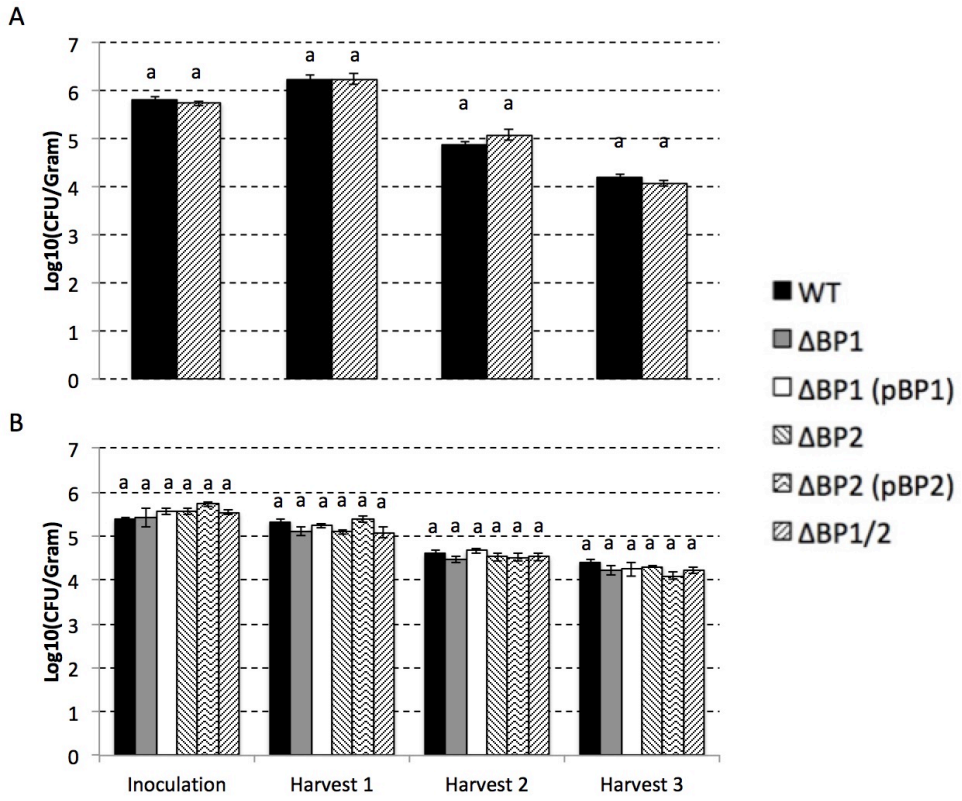


Figure 3.4. Bacterial populations in the bulk soil after repeated harvest cycles in autoclaved and natural soil. In addition to rhizosphere populations (see Fig. 3.3), bacterial populations in the bulk soil from the same repeated harvest assays were estimated. (A) Bacterial populations in autoclaved soil and (B) and natural soil. Data points represent the means from 10 biological replicates and error bars indicate standard errors. The experiment was repeated twice with similar results. For each harvest cycle, values with different letters are significantly different.

Discussion

Previous analysis of the *P. chlororaphis* 30-84 R-tailocin gene cluster revealed that it encodes two distinct R-tailocins, similar to other sequenced *P. chlororaphis* and some *P. fluorescens* strains, and that both tailocins are released by a shared lysis

cassette. The two particles have different killing spectra and appear to have different ancestry (76). Also similar to other sequenced *P. chlororaphis* strains (e.g., *P. chlororaphis* O6 and PA23) the genetic module encoding the second R-tailocin contains two different genes annotated as tail fibers. In contrast, in other species with gene clusters that are closely related to *P. chlororaphis* 30-84 tailocin 2 (e.g., *P. syringae* pv. *syringae* B728a, *P. protegens* Pf-5, and *P. chlororaphis* UBF2), the cluster contains only one gene annotated as a tail fiber (15, 78). In the present study, we found that the *P. chlororaphis* 30-84 tailocin 2 genetic module produces two independent tail fibers that incorporate separately onto the tailocin 2 particles, resulting in distinct tailocin 2 particles with different killing spectra. We speculate this may be true for other *P. chlororaphis* strains that have two tail fiber-like genes in their tailocin-2-like genetic module. Although this may be the first example of an R-tailocin having a broadened target spectrum as a result of having two different tail fiber proteins, other strategies to broaden the killing spectrum of R-tailocin particles have been reported. For example, a previous study found that the bactericidal spectrum of the phage tail-like bacteriocin carotvoricin, produced by *Pectobacterium carotovora,* was increased as a result of modification of the receptor-interacting portion of the tail fiber. This modification was mediated by an invertase encoded downstream of the tail fiber gene that altered the DNA encoding the receptor (22). Similarly, studies in bacteriophage have demonstrated that incorporation of two tail fiber proteins (110) or two tail spike proteins (111) onto the bacteriophage particle increases host specificity. It is unknown how many R-tailocin tail fibers are required for efficient binding to a susceptible cell and subsequent killing. A

hybrid tailocin that incorporates each tail fiber type may be unable to kill susceptible cells because the tail fiber interaction is not sufficient to attach to cells and trigger contraction. Utilization of two different tail fiber proteins represents a novel strategy to increase R-tailocin killing spectrum and may be an adaptation by *P. chlororaphis* strains to life in the rhizosphere microbiome.

Despite the potential for targeting a broad range of competitors via the diversity of tailocin particles produced, the relative ecological importance of the two tailocins was unclear. In the present study, a collection of culturable wheat rhizosphere bacteria isolates was screened for tailocin susceptibility. Although both tailocins targeted some of the rhizosphere isolates, the majority of susceptible strains were targeted by tailocin 2, and this was consistent among collections from different geographic locations. As expected, the R-tailocins produced by *P. chlororaphis* 30-84 inhibited some (~27%), but not all of the isolates that were fluorescent on KMB (indicative of their being pseudomonads). Interestingly, some (~8%) of the isolates that were not fluorescent on Kings Media B were targeted, suggesting that some of the targeted strains may not be pseudomonads. It is generally thought that R-tailocins target a narrow spectrum of bacteria closely related to the producer (9), but studies have found bacteria that are distantly related to the producer and occupy similar habitats are also targeted by R-tailocins. For example, the killing spectrum of the R-tailocins produced by *P. chlororaphis* and *P. fluorescens* includes fluorescent *Pseudomonas* and non-fluorescent *Xanthomonas sp.*, both of which are found in relatively high abundance on plants (50, 58, 60, 76, 112, 113). Similarly, R-tailocins produced by *P. aeruginosa* target distantly

related human pathogenic bacteria *Neisseria gonorrhea, N. meningitidus, Haemophilus ducreyi,* and *Burkholderia cepacia* (46, 114-116). Identification of the non-fluorescent strains targeted by *P. chlororaphis* 30-84 R-tailocins is ongoing.

This study expands on previous findings regarding the importance of R-tailocins in pairwise competition with sensitive strains (26, 27, 51, 52, 76) and demonstrates that R-tailocins contribute to the competitive survival of *P. chlororaphis* 30-84 in the wheat rhizosphere microbiome, the niche from which it was isolated. This discovery was facilitated by using repeat harvest cycles in both autoclaved and non-autoclaved wheat field soil to rapidly amplify the outcome of competition across plant growth cycles. In autoclaved soil in which a large proportion of the indigenous microorganisms were inactivated, competition between the remaining indigenous wheat rhizosphere microorganisms and the introduced *P. chlororaphis* 30-84 strains (e.g., wild type, mutants, or complements) should be significantly less than in non-autoclaved soil. In non-autoclaved soil, superior competitors should maintain higher populations than weaker competitors as selection among wheat root colonists intensifies with repeated harvest cycles. In non-autoclaved soil, the tailocin producer populations were larger than the non-producers after the second and third harvest cycles, whereas in the autoclaved soil both tailocin producers and non-producers reached similar, higher population densities.

Despite the importance of tailocin production for wheat rhizosphere persistence, we were not able to measure a similar contribution to bacterial persistence in bulk soil. We found that producers and non-producers attained similar population densities in both

non-autoclaved and autoclaved soil, albeit reaching slightly higher densities in autoclaved soil. This may be due to differences in the relative abundance and metabolic state of bacteria in bulk soil as well as the physical properties of this environment. For example, tailocin production may be a more important competitive strategy in the rhizosphere where higher nutrient availability results in larger population densities within localized niches (e.g., within the rhizoplane and immediate surroundings) leading to more cell-to-cell interaction as compared to bulk soil. Moreover, as compared to bulk soil, rhizospheres have been shown to be enriched for Proteobacteria (*Pseudomonas* and *Xanthomonas* spp.), known targets of R-tailocins produced by plant-associated pseudomonads (59, 60, 112, 113, 117). Adherence to soil particles as well as the metabolic state of the cells may have affected particle infectivity in bulk soil. Although the influence of soil properties on the activity of tailocin particles has not been studied, the effect of soil particles on bacteriophage infectivity has been studied (118). For example, it was shown that clay particles promote the adsorption of some phage particles, reducing phage infectivity (118). Additionally, it was hypothesized that bacteria may be insensitive to phage infection in soil because the phage receptor is not expressed in the metabolic state supported by the soil environment (118). Lipopolysaccharides (LPS) are commonly the receptor targeted by tailocin particles (10, 31, 45, 54) and LPS biosynthesis is altered by growth phase and nutrient availability (119-121). In our study, a predominantly clay soil was used and the extent to which this may have affected either tailocin activity or target sensitivity is unknown. Future studies should focus on understanding how soil properties influence tailocin killing activity.

Plant rhizospheres are highly variable landscapes supporting different niches that are occupied by rich and dynamic microbial communities (56, 122). Roots are composed of zones that differ in cell maturation, provide distinct functions, and support discrete microbial populations (122). The spatial distribution and size of bacterial communities varies along the root zones, with the largest populations associated with the root tip region (122). As the root grows and elongates through the soil, the region that was root tip matures. Thus, the bacterial communities colonizing the root tip will be exposed to changing environmental conditions associated with transport through the soil and root structural and functional maturation. Shifts in microbial population dynamics may be expected (56, 122) and as a result, rhizobacteria may need to employ different competitive strategies to survive in rhizosphere communities. This study reveals the importance of one competitive strategy, R-tailocin production, for bacterial persistence in rhizosphere communities.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Plant growth promoting rhizobacteria (PGPR) are a conspicuous group of rhizosphere inhabiting organisms that provide benefits to the host plant in multiple ways including: facilitating plant acquisition of nutrients, protecting plants against pathogens, enhancing plant tolerance of abiotic stresses, and improving plant growth and fitness. Increasingly, inoculations of PGPR are being considered as options for improving soil fertility and plant productivity and providing biological control capabilities; typically the best results are obtained using isolates or mixtures of isolates that are capable of providing more than one of these functions (123). An important but often overlooked requirement of effective PGPR is their ability to compete with the existing microbial communities into which they are inoculated and establish sufficient rhizosphere populations to produce the sought benefits. This is likely to be one of the primary factors contributing to instances where deployment of PGPR in the field has yielded mixed results (63, 123).

Bacteria employ many types of interference strategies to compete with other bacteria for common resources, including the production of diffusible antimicrobial

compounds, such as phenazines and bacteriocins, and contact-dependent mechanisms, such as type six secretion systems (66, 75, 124, 125). This dissertation focuses on phage tail-like bacteriocins called tailocins, which are narrow spectrum bactericidal proteins produced by a variety of Gram negative and Gram positive bacteria (9). The extensive documentation of tailocins within the genomes of PGPR *Pseudomonas* species (14, 15, 32, 33) suggests that bacteriocins may be important for their competitive fitness in plant-associated niches. The chapters within this dissertation detail the characterization of R-tailocin production by the PGPR *Pseudomonas chlororaphis* subsp. *aureofaciens* 30-84 as well as elucidate the function of R-tailocins in the ecology of *P. chlororaphis* 30-84 in competition with the rhizosphere microbiome.

In chapter II, I demonstrated that *Pseudomonas chlororaphis* 30-84 produces two functional R-tailocin particles that differ in their killing spectrum and likely also in their ancestral origin. I show that both tailocins are released by the same lysis cassette and that this unique two-tailocin / one lysis cassette arrangement is characteristic of most of the sequenced *P. chlororaphis* species, but is less commonly found among in the genomes of other sequenced pseudomonads. Results demonstrate that the production of R tailocins gives *P. chlororaphis* 30-84 a competitive advantage over a sensitive strain in pairwise competition assays when grown in biofilms and on roots. The chapter in its entirety was published during my graduate studies (76). Specific results include:

- Analysis of the *P. chlororaphis* 30-84 R-tailocin gene cluster and those of sequenced *P. chlororaphis* and *P. fluorescens* strains revealed that most *P. chlororaphis* and some *P. fluorescens* R-tailocin gene clusters contain the structural components to

produce two R-tailocins. Phylogenetic analysis suggested that the tailocin structural components have different ancestral origin. The genetic module encoding the first R-tailocin in the *P. chlororaphis* 30-84 gene cluster is most similar to R-tailocins produced by *P. fluorescens* (R-fluorescins), which appears to be ancestrally related to the *Vibrio parahaemolyticus* and *Halomonas aquamarina* phages VP882 and phiHAP-1, respectively (126, 127). Whereas the genetic module encoding the second R-tailocin is similar to R-syringacins, R-tailocins produced by *P. syringae*, and appears to be ancestrally related to the *Shigella flexneri* phage, SfV (128). Previous studies identified two R-tailocin genetic modules in the R-tailocin gene clusters of *P. fluorescens* and *P. chlororaphis* O6 (33). My study expands on previous research by including more *P. chlororaphis* and *P. fluorescens* strains. These results suggest that most sequenced *P. chlororaphis* strains can produce two different R-tailocin particles, whereas the genomes of most of the other plant-associated *Pseudomonas* that have been sequenced (i.e., *P. putida*, *P. fluorescens, P. protegens, P. syringae*) suggest they produce only one. The production of two tailocins is likely to broaden the spectrum of bacterial competitors killed by *P. chlororaphis* R-tailocins, which in turn likely enhances competitive survival in diverse microbial communities such as the rhizosphere microbiome.

- Examination of *P. chlororaphis* 30-84 UV induced lysates with a transmission electron microscope revealed two distinct R-tailocin populations based on size. Mutation of each R-tailocin baseplate components demonstrated that the first R-tailocin encodes the larger the two particles observed in the electron micrographs. Both tailocins target *Pseudomonas* whereas tailocin 2 was found to also kill *Xanthomonas*. The spectrum of

pseudomonads killed by the two R-tailocins differed and a few *Pseudomonas* species were either killed by or insusceptible to both tailocins. This study is the first demonstration that a plant-associated *Pseudomonas* species produces more than one functional R-tailocin particle. Previous work in *P. aeruginosa* found that some strains produce R- and F- type tailocin particles (11). Given that the known killing spectrum of most plant-associated *Pseudomonas* strains is limited to *Pseudomonas* species, it was interesting that a *Xanthomonas* strain was susceptible to tailocin 2. This previously observed limitation may be a consequence of the small collection of strains, comprised mostly of *Pseudomonas,* that were screened for tailocin susceptibility in these studies (14, 15, 19). A recent study in *P. fluorescens* SF4c, which possess a R-tailocin gene cluster similar to *P. chlororaphis* (i.e. two R-tailocin genetic modules), also targets *Xanthomonas* (50, 76). R-tailocins produced by *P. aeruginosa* have been shown to target other more distantly related pathogenic bacteria, such as *Neisseria gonorrhea, N. meningitidus, Haemophilus ducreyi,* and *Burkholderia cepacia* (46, 114-116). I suspect that the *P. chlororaphis* and *P. fluorescens* strains that possess two distinct tailocin structural genetic modules in their R-tailocin gene cluster also produce two R-tailocins with different killing spectra, although the killing spectra of these particles may differ from those produced by *P. chlororaphis* 30-84.

- The *P. chlororaphis* 30-84 R-tailocin gene cluster encodes a lysis cassette with a similar organization as those previously described in other plant associated *Pseudomonas* spp. (11, 14). The function of the holin and endolysin were confirmed using mutational and overexpression analysis. Tailocin release was disrupted by deletion

of the holin gene within the tailocin gene cluster, demonstrating that the lysis cassette is required for the release of both R-tailocins. These findings are consistent with the current model of tailocin-associated cell lysis (12).

- Pairwise replacement series competition assays demonstrated that the loss of functional tailocin production eliminated the competitive advantage of *P. chlororaphis* 30-84 over an R-tailocin sensitive strain within surface attached biofilm and rhizosphere communities. This was the first study to document the function of R-tailocins in competitive interactions between rhizobacteria in surface-attached biofilm and rhizosphere populations. These results are consistent with similar studies that examine the role of tailocin production in pairwise competition assays between producer and sensitive strains under different conditions, such as *in vitro* co-culture and biofilms as well as within insect and mammalian hosts (26, 27, 45, 51, 52).

In chapter III, I demonstrate the functionality of two tail fiber genes found within the gene cluster of tailocin 2 and demonstrate that they incorporate independently and separately onto tailocin 2 particles, resulting in distinct tailocin 2 particles having different tail fibers with discrete killing spectra. My findings indicate that this two-tail fiber feature of tailocin 2-type genetic modules is common to other *P. chlororaphis* strains but not to the tailocin 2-type genetic modules of other species. This is a previously unreported observation. I demonstrate that the *P. chlororaphis* 30-84 R-tailocins targeted about 12% of our collection of culturable rhizosphere isolates obtained from different geographic regions and that tailocin 2 typically had the broader killing

spectrum. Of significance, my results demonstrate that R-tailocin production enhanced persistence of *P. chlororaphis* 30-84 in the wheat rhizosphere, but that this enhancement in population size was not evident in the wheat rhizosphere when the populations of microbes in soil were reduced by autoclaving. Moreover, I was not able to measure an enhancement in competitive fitness in bulk soil and speculate this may be due to reductions in the numbers of potential competitors, their metabolic state, or other edaphic factors. The work presented in this chapter has been submitted for publication. Specific findings include:

- Genomic analysis of the *P. chlororaphis* 30-84 R-tailocin gene cluster in chapter II uncovered the presence of two tail fiber genes in the tailocin 2 genetic module. This feature is also present within the tailocin-2 type genetic modules present in other sequenced *P. chlororaphis* strains (e.g., *P. chlororaphis* O6 and *PA23*). In contrast, in other species with genetic modules that are closely related to *P. chlororaphis* 30-84 tailocin 2 (e.g., *P. syringae* pv. *syringae* B728a, *P. protegens* Pf-5, and *P. chlororaphis* UBF2), only one gene annotated as a tail fiber is present (15, 78). In this study, the two tail fibers were found to incorporate onto different tailocin 2 particles, each with distinct killing spectra. This is a previously unreported mechanism that results in a broader killing spectrum for a single R-tailocin although other mechanisms that broaden the host killing spectra of tailocins or phages have been described (22). The mechanism by which only one of the tail fibers is incorporated onto tailocin 2 is unknown, but may be associated with differences in the gene expression or function of the different chaperone proteins associated with each tail fiber, although at present there is no evidence for either

hypothesis. The tail fiber chaperone proteins are required for efficient attachment to the baseplate, however the mechanism by which this occurs remains to be elucidated (9, 31). Regardless of the mechanism, the expanded killing spectrum afforded to tailocin 2 by the two tail fibers may enhance the competitive capacity of *P. chlororaphis* 30-84 in the rhizosphere microbiome.

- The breadth of influence of the R-tailocins on native wheat rhizobacteria was examined by screening a collection of wheat rhizobacteria (484 strains) for susceptibility to either tailocin. Nearly 12% of the collection was targeted by one of the tailocins, the majority being susceptible to tailocin 2. Interestingly, isolates that did not fluoresce when grown on King's B Media (fluorescence is indicative of fluorescent pseudomonads) were targeted by *P. chlororaphis* R-tailocins, suggesting that some of the susceptible isolates may not be pseudomonads. It is generally thought that R-tailocins target only a narrow spectrum of bacteria closely related to the producer (9), but studies have found bacteria that are distantly related to the producer and occupy similar habitats are also targeted by R-tailocins (46, 50, 114, 116). Some R-tailocin particles appear to target both strains that are closely related to the producing strain and also target more distantly related strains that inhabit similar niches as the producer.
- Loss of production of one or both tailocins resulted in decreased *P. chlororaphis* 30-84 persistence within the wheat rhizosphere when in competition with the native microflora, but not bulk soil. This may be a consequence of the differences in the relative abundance and metabolic state of bacteria in bulk soil as well as the physical properties of this environment. For example, tailocin production may be a more

important competitive strategy in the rhizosphere where high nutrient availability facilitates the growth of large bacterial populations. In the rhizosphere, the altruistic act of tailocin production may be favored because cells are actively growing and are in close proximity to competitors that could encounter R-tailocin particles. Whereas in bulk soil, the cells are not actively growing and may not be within the vicinity of competitors that will contact R-tailocin particles. Moreover, as compared to bulk soil, rhizospheres have been shown to be enriched for Proteobacteria (*Pseudomonas* and *Xanthomonas* spp.), known targets of R-tailocins produced by plant-associated pseudomonads (59, 60, 112, 113, 117). Adherence to soil particles as well as the metabolic state of the targeted cells also may affect particle infectivity in bulk soil. Although the influence of soil properties on the activity of tailocin particles has not been studied, the effect of soil particles on bacteriophage infectivity has been studied (118). For example, it was shown that clay particles promote the adsorption of some phage particles, reducing phage infectivity (118). Additionally, it was hypothesized that bacteria may be insensitive to phage infection in soil because the phage receptor is not expressed in the metabolic state supported by the soil environment (118). Lipopolysaccharides (LPS) are commonly the receptor targeted by tailocin particles (10, 31, 45, 54) and LPS biosynthesis is altered by growth phase and nutrient availability (119-121).

The results presented within my dissertation are the first to demonstrate that R-tailocin production enhances the competitive fitness of a PGPR within the community of organisms comprising the natural microbiome of the wheat rhizosphere. The widespread prevalence of genes encoding R-tailocins within the genomes of other sequenced PGPR

and plant pathogens suggests this competitive mechanism may be widely employed by plant-colonizing microbes. Previous studies have documented interference mechanisms employed by PGPR that enhance their competitive capacity, including the production of antimicrobial secondary metabolites such as phenazines, hydrogen cyanide, and 2,4, diacetylphloroglucinol (75, 129) as well as the utilization of contact dependent mechanisms like type six secretion systems (124, 125, 130). I believe the multiplicity of mechanisms within the armory of plant-associated microbes likely reflects both the taxonomic diversity of competitors and the dynamic variety in the niches for which these microbes must compete. Thus, PGPR inoculants should be selected for having multiple plant-beneficial functions as well as a diverse antimicrobial arsenal to insure the establishment or adequate rhizosphere populations to yield the desired plant-health benefits.

Future Work

Chapter II:

- Characterize the biocontrol potential of Tailocin 2, which was found to target phytopathogenic *Pseudomonas syringae* and *Xanthomonas campestris* strains. This includes: 1) determining whether R-tailocin particles remain stable on plant surfaces over time, 2) whether R-tailocins are able to kill bacteria when applied

epiphytically or directly infused into the apoplast, 3) characterize the fitness of spontaneous tailocin resistant strains *in vitro* and *in planta*, 4) determine whether R-tailocins interact with the plant immune system.

- Identify the cell surface receptors targeted by the tail fibers associated with tailocin 1 and both tailocin 2 particles. This information will be useful when developing tailocin cocktails to treat bacterial infections. Using R-tailocins that target distinct receptors decreases the likelihood of the spontaneous emergence of resistant bacteria.
- Determine the number of each R-tailocins particles produced per cell (burst size). This information can be used when developing R-tailocin cocktails to treat bacterial infections.
- Characterize the tailocins produced by other rhizosphere-associated *Pseudomonas*. Determine the killing spectra of these particles as well as identify the receptors targeted that these particles. These data may also be used to predict the killing spectra of uncharacterized R-tailocin particles by comparing the receptor interacting region (C-terminal of tail fiber protein) amino acid sequences of characterized R-tailocins to uncharacterized R-tailocins.

Chapter III:

- Elucidate the mechanism by which each tail fiber is attached to the tailocin 2 particle. Understanding this mechanism provide important insight to the assembly of R-tailocin particles and may provide information about better strategies to engineer receptor binding proteins.

- Identify the tailocin susceptible isolates that did not fluoresce when grown on KMB media. This information is important for understanding the complete range of culturable rhizosphere bacteria targeted by *P. chlororaphis* 30-84 R-tailocins.
- Determine whether the application of R-tailocin solutions to plant-less or plant-containing soils perturbs plant-associated bacterial community composition. This information would be useful for understanding the potential of using R-tailocins to manipulate plant-associated bacterial populations and remove harmful species.
- Understand whether soil properties influence killing activity of R-tailocin particles. This information would be useful to understanding efficacy of R-tailocin application in soil-ecosystems.
- Identify the niches wherein R-tailocin production is most effective on roots. This would include; 1) characterize the regions on roots where R-tailocins are produced, 2) determine the type of populations that produce R-tailocins (i.e. size and location), 3) identify where and when tailocin production is most effective on plant roots (i.e. location, mixed species vs. non-mixed populations). This information would provide important information about how R-tailocin production influences bacterial population dynamics on plant roots.

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APPENDIX

PSEUDOMONAS CHLORORAPHIS 30-84 SMALL COLONY VARIANTS

Introduction

This Chapter describes aspects of my lab rotation project in the Pierson lab, which included experiments that were part of a larger project on characterizing the genetic and transcriptomic alterations associated with small colony variant phenotypes in the plant-beneficial bacteria species, *Pseudomonas chlororaphis* 30-84. This work resulted in the following peer reviewed publication: Wang D, Dorosky RJ, Han CS, Lo C, Dichosa AEK, Chain PS, Yu JM, Pierson LS, III, Pierson EA. 2015. Adaptation genomics of a small-colony variant in a *Pseudomonas chlororaphis* 30-84 biofilm. *Applied and Environmental Microbiology* 81:890–899. doi:10.1128/AEM.02617-14. Below I provide a brief introduction to the paper and an overview of significant findings as well as describe my contributions to the published work and additional experiments I performed.

When adjusting to the biofilm lifestyle, some *Pseudomonas* undergo phenotypic diversification (1, 2). Small colony variants (SCV) phenotypic variants that are routinely isolated from *Pseudomonas* biofilms and exhibit slow growth, hyper adherence, and antimicrobial resistance (1, 3-5). Recent studies found that SCV phenotypes are

associated with elevated cellular pools of cyclic-di-GMP (c-di-GMP)(6-9). In *P. aeruginosa* PAO1, deletion of the c-di-GMP regulator, *yfiR,* resulted in elevated c-di-GMP and the formation of SCVs, which indicated a central role of c-di-GMP in SCV emergence and phenotypes (7). YfiR is one of two negative regulators of YfiN, a diguanylate cyclase that was originally named TpbB (10). YfiN is also regulated by the phosphataseTpbA. Inactivation of TpbA results in increased YfiN activity and the formation of SCVs (10). These studies demonstrated the link between the YfiNBR pathway, intracellular c-di-GMP levels, and the SCV phenotype in *P. aeruginosa*.

In our study, we phenotypically, genomically, and transcriptionally characterized a SCV that was isolated from a *P. chlororaphis* 30-84 biofilm. The *P. chlororaphis* 30-84 SCV exhibited phenotypes characteristic of SCVs previously described in other *Pseudomonas* stains, including small rugged colonies, small cell morphology, slow growth rate, heightened adherence, elevated antimicrobial resistance, and reduced motility (3, 7, 11). All of these phenotypic alterations are consistent with elevated cellular pools of c-di-GMP (8, 9, 12-14). Genomic and transcriptional analyses identified specific alterations associated with the SCV phenotype and two of the major genetic alterations were characterized. One of these alterations was a 33-bp deletion within the coding region of a gene with similarity to the c-di-GMP regulator, *yfiR*. The second was a single nucleotide polymorphism within the coding region of *fusA*, the gene encoding elongation factor G (EF-G).

My contribution to the paper was to use molecular cloning and gene expression to determine the contribution of each mutation to the SCV phenotypes. I found that *in*

trans complementation of the SCV with the wild type *yfiR* restored most of the SCV phenotypes (15). The colony and cell morphology as well as motility and growth rate phenotypes were restored to wild type levels with the introduction of the wild type *yfiR*. Kanamycin susceptibility was only partially restored by complementation with wild type *yfiR*, indicating that the other alterations may be involved in the kanamycin resistance phenotype. Kanamycin inhibits protein synthesis by binding the 30S ribosomal subunit and inhibiting ribosome translocation and proofreading capabilities (16). EF-G is involved in the translocation of the ribosome during protein synthesis and mutations to EF-G may alter kanamycin activity. Introduction of the SCV copy of *fusA* into wild type increased its kanamycin resistance, which suggested the genetic alteration to *fusA* partially contributed to the kanamycin resistance phenotype of the SCV. Mutations to EF-G have previously been shown to lead to slight increases in kanamycin resistance (17, 18). These data demonstrated that the majority of the SCV phenotypes were associated with the mutation to *yfiR* and that the mutations in both *yfiR* and *fusA* contribute to the enhanced kanamycin resistance of the SCV. Thus, my contributions to the published research included some phenotypic characterization of the *P. chlororaphis* 30-84 SCV and the determination of the genetic changes responsible for the major SCV phenotypes.

In addition to my contributions to the published work, I initiated other studies focused on: 1) characterizing SCV biofilm matrix production, 2) evaluating the fitness of SCVs in plant-associated environments, and 3) understanding the contribution of the GacS/GacA two component transduction system (TCTS) to SCV emergence and

phenotypic switching under different growth conditions. I describe the methods and findings of these additional studies and briefly discuss their significance below.

Experimental Procedures

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are described in Table A.1. A spontaneous rifampin-resistant derivative of *P. chlororaphis* 30-84 was used in all studies. The *P. chlororaphis* SCV used for this study were isolated from biofilm cultures of wild type *P. chlororaphis* 30-84 (15). LB medium or AB minimal medium supplemented with 2% casamino acids (AB + 2% CAA) (Difco, Becton Dickinson and Company, Franklin Lakes, NJ) were used for culturing *P. chlororaphis* as described previously (15, 19).

Table A.1. Bacterial Strains and Plasmids used in this appendix.

Strain	**Description/Source**	**Reference**
P. chlororaphis 30-84	Wild Type, Rif^R	W.W. Bockus
P. chlororaphis 30-84 SCV	Spontaneous mutant	(15)
P. chlororaphis 30-84 GV	Spontaneous Gac/Rsm deficient mutant. Mutation to GacA	(2)
Plasmid	**Description/Source**	**Reference**
pGT2	Gm^R, GFP-based promoter trap vector containing a promoter less *gfp* gene	(20)
pYfiR	pGT2 containing fragment containing YfiR coding sequence	(15)

Quantification of biofilm matrix and eDNA. For biofilm cultures, bacteria were grown in plates without shaking using an established method with modifications (21, 22). Pre-cultures were prepared overnight in shaking glass tubes (200 rpm) filled with 3 mL AB + 2% CAA at 28°C. The pre-cultures then were diluted to an OD_{600} of 0.05 in AB + 2% CAA and 1.5 ml per well were added to 12 wells of 24-well polystyrene Corning® Costar® cell culture plates (Corning Inc., Corning, NY, USA). The biofilm plates were sealed with an air-permeable cover and placed in an incubator at 28°C for up to 72 h. At 72 hours post inoculation, the entire non-adhering 1.5 mL static cultures were transferred to Eppendorf tubes and cells collected by centrifugation (16,000 x g for 5 min). The supernatants were removed and the mass of the cells and hydrated matrix were measured. The concentration of extracellular double stranded DNA was determined quantitatively using a Qubit 2.0 Fluorometer (Invitrogen Life Technologies, CA, USA). Briefly, static cultures were diluted 1:10 in water and vortexed

to thoroughly disrupt the EPS. After centrifugation (16,000 x g for 5 mins), the supernatants were transferred to new tubes and filter sterilized (0.22 μm filter, Millipore, MA, USA). The concentration of eDNA was quantified by mixing 10 μl of supernatant with DNA quantifying fluorescent dyes from Qubit (Invitrogen Life Technologies). The fluorescence of DNA-dye interaction was measured using a Qubit 2.0 Fluorometer according to the manufacturer's instructions. The amount of eDNA was determined via comparison to a standard curve and reported as μg/ml.

Seed Adherence. Wheat seeds (TAM304) were surface disinfested and coated with wild type *P. chlororaphis* 30-84 or the SCV (15) as described previously (23). Briefly, bacteria were grown overnight in AB+ 2% CAA, resuspended (OD_{620}=0.5), and diluted 1/50 in 125mL beakers containing 10mL AB+ 2% CAA and 8 disinfested seeds. The seeds and bacteria were incubated for 96 hours. After 96 hours, the seeds were washed 3 times with sterile water and then placed into 2 mL of sterile water. Bacteria were isolated by vortexing (10 s) and sonication (10 s) three times. Bacterial populations were determined by serial dilution plating on LB agar.

Rhizosphere Colonization. To better understand how the SCV behaves in soil-rhizosphere ecosystems a rhizosphere colonization assay was conducted. Wild type *P. chlororaphis* 30-84 or the SCV (15) were inoculated into autoclaved (45 min. twice with a 24 h break) wheat field soil (Leskovar, Texas) and sown with disinfested wheat seedlings (TAM304) and grown for 30 days (dark (8hr) and light (16hr) cycle at 27 ± 2°C. Bacteria were grown overnight, washed twice in sterile water, and resuspended in sterile water (20mL) to a final concentration of $1x10^9$ CFU/mL. This suspension was

mixed with soil to a final concentration of 10^7 CFU/g by diluting the suspension ten-fold in 20mL (sterile water) and then adding the diluted suspension to soil (400g) and mixing thoroughly. Wheat seeds (TAM304) were surface disinfested by incubation in 70% ethanol for 10 minutes followed by incubation in 90% bleach. Disinfested seeds were pregerminated on seed paper for two days and the seedlings were sown in the bacteria infested soil four days after soil inoculation. After 30 days of growth, plants from each soil treatment were harvested and sampled for bacterial population sizes, as previously described (24, 25). Briefly, the whole root system of each plant was collected, placed in 5ml of phosphate buffered saline (pH 7.4), and sonicated (3x) and vortexed (3x) for 10 seconds. Bacterial suspensions were serially diluted and plated onto LB supplemented with Rif. The roots were dried for 48 hours in a 65°C oven and populations were standardized to dry root weight.

SCV emergence assay. To better understand the role of the Gac/Rsm signal transduction system in the emergence of SCV in *P. chlororaphis* 30-84, wild type or ΔGacA shaking and biofilm cultures were analyzed for the emergence of SCVs. Briefly, bacterial strains were grown overnight in AB + CAA at 28°C with agitation (200 rpm), harvested, washed, and resuspended in fresh medium before creating the starting cultures. Cultures were standardized to OD_{620}:0.1 and diluted (1:100) into 15-ml polypropylene tubes containing AB minimal media supplemented with 2% cassamino acids (3 ml). The polypropylene tubes were incubated at 28°C with shaking (200 rpm) or without shaking for the shaking and biofilm conditions, respectively. After 24, 48, and 72 h, the liquid and loosely adherent cells were removed by pipetting. The surface

attached biofilm was washed three times with sterile water. After washing, 5 ml of phosphate buffered saline (pH 7.4) was added and the tubes were sonicated (3x, 10 sec) and vortexed (3x, 10 sec) to remove surfaced-adhered bacteria. Population sizes were quantified by dilution plating. Serial dilutions were plated on LB agar where *P. chlororaphis* 30-84 SCVs can be readily identified. After two days of growth at 28C, the plates were examined for SCVs. If SCVs were identified, they were re-plated onto a new plate to confirm the phenotype is stable.

Results and Discussion

SCV biofilms produce smaller biofilm matrices that contain less eDNA. Our previous study found that the SCV forms larger surface attached biofilms than the wild type strain from which it was derived and this enhanced biofilm capability was due to spontaneous mutation in the coding region of the c-di-GMP regulator, *yfiR* (15). My rotation research expanded on the previous observations by examining the characteristics of the SCV in floating biofilms as opposed to surface attached biofilms. Study of floating biofilm cultures provides the opportunity to both measure biofilm matrix production and the amount of extracellular DNA (eDNA) that holds it together (22). The biofilm matrix is composed of self-produced extracellular polymeric substances that surround bacterial cells, provide structure to the community, and influence the

physiochemical properties within biofilms (26, 27). Although the capsule surrounding the SCV cells was thicker, the floating biofilm matrix formed by the SCV after 72 h was significantly smaller than that of wild type (Fig. A.1A). In fact, the biomass of the SCV biofilms appeared to accumulate almost solely in surface attached populations, whereas the biomass of wild type biofilms was distributed in between surface-attached and non-attached biofilms. The observed defect in floating biofilm matrix biomass accumulation was partially restored by complementation of the SCV with the wild type copy of the negative regulator of c-di-GMP accumulation, *yfiR.* This indicates that the SCV impairment in floating biofilm matrix accumulation is likely a consequence of the enhanced intercellular pools of c-di-GMP in the SCV. Given the hyper surface-attached phenotype of the SCV biofilm, it is not surprising that the SCV floating biofilm features are less prominent than those in the wild type. A major component of bacterial biofilm matrices is eDNA, which acts as a scaffolding that gives structure to *P. aeruginosa* and *P. chlororaphis* biofilms (22, 28). I expected to observe less eDNA in the SCV floating biofilms than the wild type because of the reduced floating biofilm matrix produced by the SCV as compared to the wild type. Not surprisingly, the SCV floating biofilm matrix accrued less eDNA than that of the wild type and this phenotype was partially restored by complementation of the SCV with the wild type version of *yfiR* (Fig. A.1B). In a recent study, it was found that phage tail-like bacteriocins mediate cell lysis and eDNA release, which was essential for establishing surface attached biofilms by *P. aeruginosa* (29). The bacteriocin cell lysis mechanism characterized in our previous study was upregulated in the SCV transcriptome (15), suggesting a role of this lysis mechanism in

the establishment of surface attached biofilms in the SCV. Future work should explore the role of this lysis system and eDNA production in surface attached biofilms formed by the SCV.

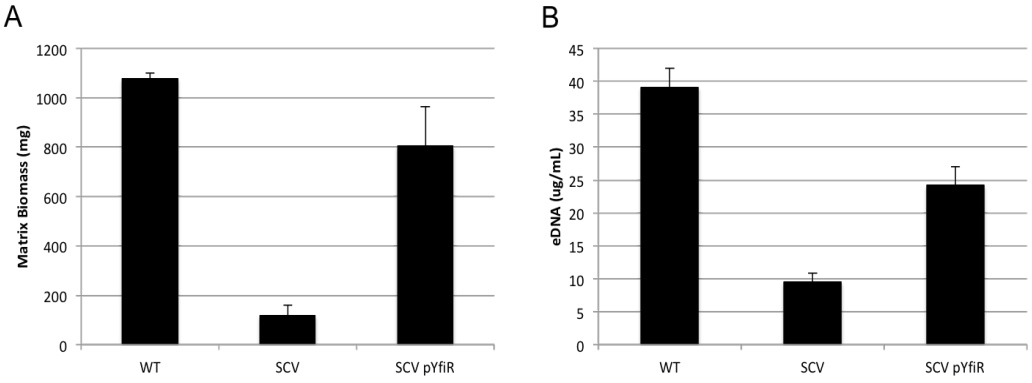

Figure A.1. Production of biofilm matrix biomass and eDNA by wild type and SCV *P. chlororaphis* 30-84. (A) Bacteria were grown in AB minimal media + 2% casamino acid in static plates for 72 h. Biofilm matrix production by wild type *P. chlororaphis* 30–84 and SCV at 72 h was quantified by weight. (B) Production of eDNA *P. chlororaphis* 30-84 wild type or SCV was quantified using the double stranded DNA quantifying fluorescent dye assay from Invitrogen. The data are the average of six biological replicates and error bars indicate the standard deviation.

Wild type and SCV exhibit similar seed adherence and rhizosphere colonization capability. The emergence and persistence of SCV in pathogenic bacterial populations is associated with antimicrobial resistance and chronic infection (11, 30, 31). Despite the occurrence of SCV in plant-associated *Pseudomonas* (15, 32), studies of these SCV have been limited to phenotypic characterization or *in vitro* ecology and

evolution studies (5, 32-35). To better understand the function of SCV in the plant-associated lifestyle of plant-beneficial *Pseudomonas,* the ability of a *P. chlororaphis* 30-84 SCV to adhere to wheat seeds, colonize wheat roots and establish in wheat rhizospheres were examined. As a first step, a seed biofilm assay (23) was conducted to better understand SCV biofilm formation on seeds. Interestingly, the SCV biofilm population sizes established on wheat seeds were not significantly different from those formed by wild type (Fig. A.2). This indicates that unlike *in vitro* surface attached biofilm assays (15), the SCV does not form larger surface attached biofilms than wild type on wheat seeds. This result may be a consequence of the difference in the physical and chemical properties of polypropylene tubes and seed coat surfaces or the duration of the two experiment (e.g., the surface attached biofilm experiments were incubated for 48 h whereas the seed biofilm assay was incubated for 96 h), the latter providing more time for biofilm formation by the wild type strain. Nonetheless my result indicates that the SCV and wild type are not different in their ability to colonize wheat seeds.

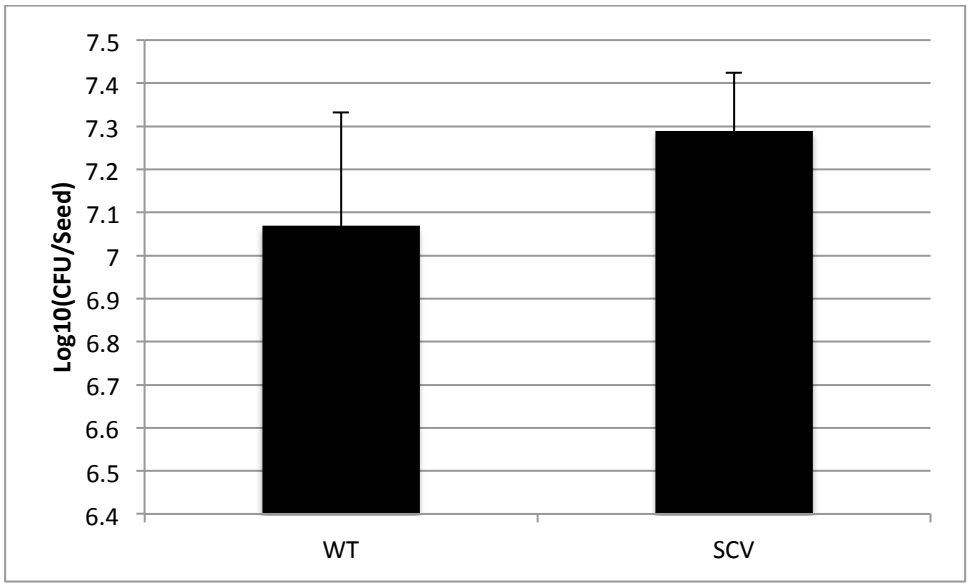

Figure A.2. Biofilm populations on wheat seeds. Seeds were treated with bacterial suspensions containing either wild type *P. chlororaphis* 30-84 or the SCV. There is no significant different in the wheat seed biofilms formed by the SCV and wild type strain. Populations are expressed as the log10 value of the CFU/seed and the error bars represent standard error. Data from only one representative experiment is given.

To better understand the ability of the SCV to colonize wheat roots and the wheat rhizosphere, a rhizosphere colonization experiment was performed. In this assay, bacteria were inoculated into autoclaved wheat field soil sown with disinfested wheat seeds. During the 30 day plant growth period, the bacteria from the soil colonized the wheat rhizosphere. After 30 days, the rhizosphere populations were quantified by dilution plating as CFU. The SCV rhizosphere populations were similar to those formed by wild type, indicating the ability of the SCV to colonize the rhizosphere is no different from the wild type (Fig. A.3). Previous work on the *P. chlororaphis* 30-84 SCV demonstrated it exhibited impaired motility (15), an important trait for colonization of

the rhizosphere (36, 37). This finding indicates that despite the impaired motility of the SCV strain, it colonizes wheat roots similar to wild type. This finding may be a result of the concentration of bacteria inoculated into the soil (10^7 CFU/gram of soil) or the length of the experiment. This inoculation rate may be too high to see small differences between strains in mobility and colonization capability. The length of the experiment also may have afforded the SCV time to replicate to populations of similar size. Future investigations should consider these factors when designing experiments. Additionally, future studies should employ repeat harvest assays to determine whether the SCV persists as well as the wild type in the rhizosphere.

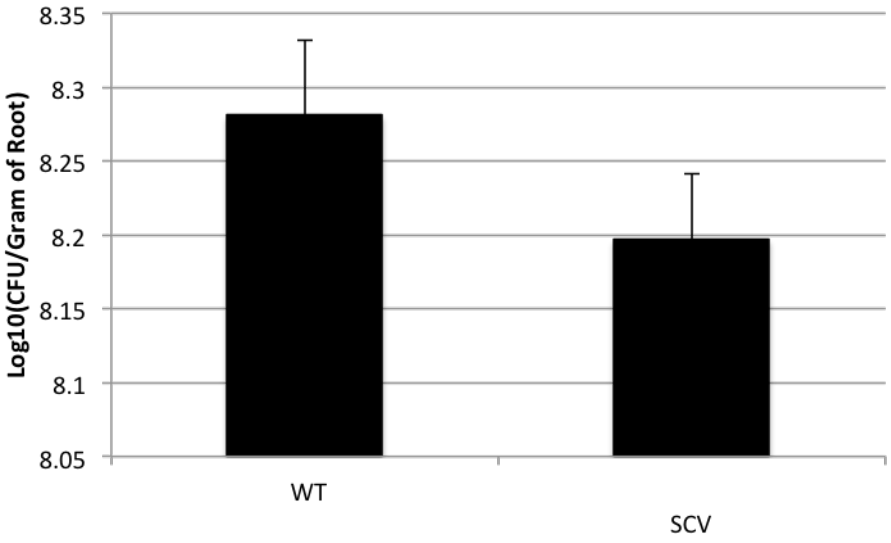

Figure A.3. Colonization of the wheat rhizosphere. Inoculum of wild type and SCV were applied into autoclaved wheat field soil (Uvalde, TX). Surfaced sterilized, pregerminated wheat seeds (TAM304) were sown into the inoculated soil and grown 30 days. The rhizosphere populations were quantified by serial dilution plating. There was no difference in the rhizosphere populations formed by the WT and SCV. Data points represent the means from 10 biological replicates and error bars indicate standard errors. The experiment was repeated twice with similar results. For each harvest cycle, values with different letters are significantly different.

Inactivation of Gac/Rsm increases the emergence of SCV in biofilm populations. The GacS/GacA is a two component transduction system that activates the production of secondary metabolites required for virulence and biocontrol activities of pathogenic and beneficial *Pseudomonas* (4, 38-44). Despite the importance of the GacS/GacA TCTS, spontaneous phenotypic variants with inactivated GacS/GacA TCTS emerge in natural populations of pathogenic and beneficial *Pseudomonas* strains (24, 45-47). In some *P. aeruginosa* strains, inactivation of the GacS/GacA TCST system increases the frequency of SCV emergence in biofilm and a mucosal surface infection model conditions (4, 48). However, the role of GacS/GacA TCTS in the emergence of SCV in plant-associated *Pseudomonas* is unknown. To address this, I measured the emergence of SCVs in shaking and biofilm cultures of *P. chlororaphis* 30-84 wild type and of a spontaneous GacA mutant (GV) (2). No SCVs were harvested from the shaking or biofilm cultures of the wild type strain at 24, 48, and 72 h (Fig. A.4). Although no SCV were harvested from wild type biofilms in this experiment, SCV are often isolated by members of our lab from wild type biofilm cultures or wheat roots. The *P. chlororaphis* 30-84 SCV described previously was harvested from a wild type biofilm grown for 96 h, suggesting a longer growth period may be required for SCV emergence in wild type biofilms. SCV were isolated from the shaking GV culture after 72 h and the SCV comprised approximately 10% of the total population. SCVs were isolated from the GV biofilm cultures after 24 h incubation, where they encompassed approximately 25%

of the population. After 48 and 72 h of incubation, the SCV comprised nearly 60% of the GV population. The SCV collected from the GV cultures retained some GV phenotypes (e.g. hyper fluorescence and no phenazine production), but also possessed stable SCV phenotypes (e.g. small wrinkly colonies, hyper biofilm formation). The emergence of SCV in GV biofilms may be a mechanism by which GV that are intrinsically more susceptible to oxidative stress than wild type cope with stress generated under biofilm conditions (4, 48). To date, there are no reports of SCV emergence in the rhizosphere populations of *Pseudomonas* species. Future work should elucidate the conditions (if any) under which SCV emergence is favored in plant-associated *Pseudomonas* populations.

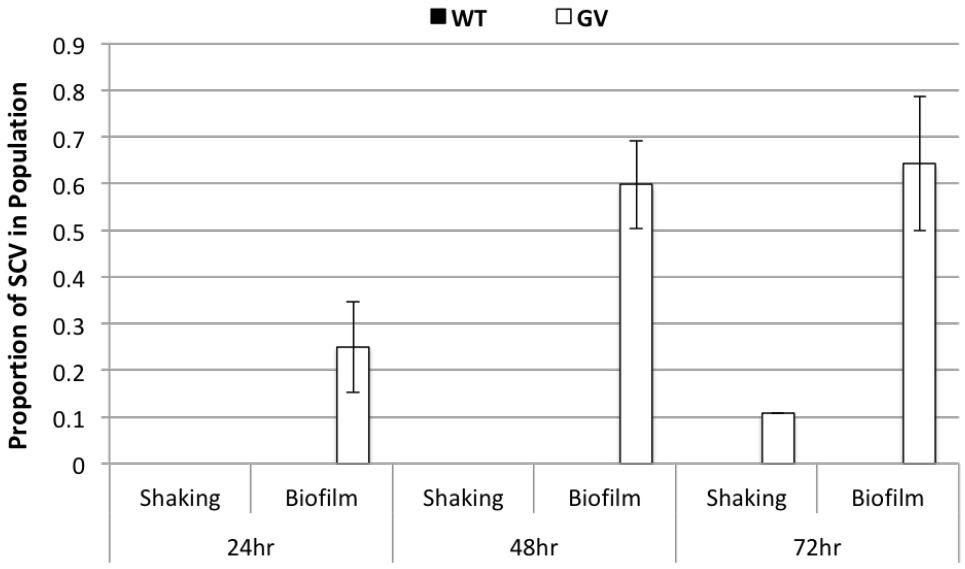

Figure A.4. Proportion of SCV isolated from shaking or biofilm (static) cultures of wild type (WT) or GacA- phenotypic variant (GV). SCV were not isolated from any of the wild type populations after 72 h of incubation. SCV were isolated from GV shaking cultures after 72 h. SCV were first isolated from GV biofilm populations after 24 h and the proportion of SCV in the biofilm increased and stabilized at 60% after 48 and 72 h, respectively. The experiment was repeat three times with five biological replicates each time. The data is a representative sample. The error bars represent the standard error of the mean.

In summary, my rotation work revealed important differences in the matrix produced by SCVs in floating biofilms as compared to wild type and indicated a role for the negative regulator of c-di-GMP accumulation, *yfiR,* in mediating matrix production. Of significance for plant-associated niches, my results indicated no impairments in the ability of SCVs to colonize and form biofilms on seed coats or to establish or persist in the wheat rhizosphere under the experimental conditions used, despite SCV impairments in motility *in vitro*. Strikingly, my work shows that Gac- phenotypic variants more

frequently give rise to SCVs and that when this occurs, these may become a relatively large proportion of the population. Overall, it is becoming recognized that phenotypic variation represents a mechanism by which bacterial species rapidly evolve distinct phenotypes in response to rapidly changing or diverse conditions (49). The importance of phenotypic variation for microbial success within the spatially and temporally dynamic niches found in the rhizosphere warrants further study.

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