Prophylactic Application of Tailocins Prevents Infection by *Pseudomonas syringae*

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Abstract

Tailocins are phage-derived bacteriocins that demonstrate great potential as agricultural antimicrobials given their high killing efficiency and their precise strain-specific targeting ability. Our group has recently categorized and characterized tailocins produced by and tailocin sensitivities of the phytopathogen *Pseudomonas syringae*, and here we extend these experiments to test whether prophylactic tailocin application can prevent infection of *Nicotiana benthamiana* by *P. syringae* pv. *syringae* B728a. Specifically, we demonstrate that multiple strains can produce tailocins that prevent infection by strain B728a and engineer a deletion mutant to prove that tailocin targeting is responsible for this protective effect. Lastly, we provide evidence that heritable resistance mutations do not explain the minority of cases where tailocins fail to prevent infection. Our results extend previous reports of prophylactic use of tailocins against phytopathogens, and establish a model system with which to test and optimize tailocin application for prophylactic treatment to prevent phytopathogen infection.

Introduction

Compared to the incredible advances that have occurred over the last century towards the clinical treatment of human and animal diseases, antibiotic treatments for prevention and control of infection of phytopathogens in plants have had relatively slower development and have been the subject of numerous debates over efficacy and effectiveness (Stockwell and Duffy 2012; McManus *et al.* 2002). Realistic options for the treatments of agricultural disease have further narrowed with heightened emphasis on the importance of one health initiatives as well as increasing recognition that broad spectrum antibiotics may have negative collateral effects on beneficial microbiota (Becattini *et al.* 2016; Robinson *et al.* 2016). With these ideas in mind we sought to develop and test for the ability of phage derived bacteriocins, which maintain a relatively precise and narrow spectrum killing activity against target strains, as a means to prevent infection by *Pseudomonas syringae.* We further describe this model system as a way to explore optimization of strain-specific prophylactic tailocin applications.

Recent efforts in the development of agricultural antimicrobials have turned towards developing specific and tailored treatments, such as the application of bacteriocins, as preventative measures for plant and animal disease (Buttimer *et al.* 2017; Rooney *et al.* 2020; Cotter *et al.* 2013; Behrens *et al.* 2017). Bacteriocins are a subset of antimicrobial compounds produced by various bacteria, which are largely thought to have a narrower spectrum of killing activity than more commonly used broad spectrum antibiotics (Chikindas *et al.* 2018; Riley and Wertz 2002). Increased specificity of bacteriocins occurs because these molecules must interact

with dedicated receptor regions or proteins on target cells before antimicrobial activity is initiated (Behrens *et al.* 2017). Phage derived bacteriocins (also known as tailocins) are a subset of bacteriocins in which the phage tail structures have been coopted by bacteria through evolution to target and kill bacteria through binding and depolarization of their membranes (Patz *et al.* 2019). Tailocins have shown potential as a platform for the development of antimicrobials with high specificity against human and animal pathogens *in vitro* and *in vivo*, with additional demonstrated capability to engineer and expand target specificity through the incorporation of tail proteins from extant phage (Scholl *et al.* 2009; Williams *et al.* 2008; Ritchie *et al.* 2011). We have recently discovered and described a tailocin locus present in the phytopathogen *P. syringae*, in which the killing specificity of the tailocins is determined by interactions between receptor binding proteins and the Lipopolysaccharide (LPS) layer of target strains (Hockett *et al.* 2015; Kandel *et al.* 2020; Hockett *et al.* 2017). A recent report demonstrated that prophylactic tailocin application could prevent infection of tomatoes by *Xanthomonas* (Príncipe *et al.* 2018), and we therefore tested whether these results could be extended to other phytopathogenic systems like *P. syringae*.

We have previously shown that tailocins from strains *Psy*Cit7 and USA011R can target strain *Psy*B728a, which can infect and cause disease in *Nicotiana benthamiana*. Here we establish that application of *P. syringae* tailocins (from strains *Psy*Cit7 and USA011R) prior to infection can prevent infection and disease in *Nicotiana benthamiana* caused by *P. syringae* strain *Psy*B728a. These results demonstrate that this protective ability is solely determined by the production and specificity of the tailocin molecules themselves and is conserved across different strains that produce tailocin molecules with similar killing spectra.

Materials and Methods

Bacterial Strains and Growth Conditions. *Psy*Cit7 was originally acquired from Steve Lindow and was described in (Lindow 1985). *P. syringae* strain USA011 was originally isolated by Cindy Morris (Morris *et al.* 2010). USA011R is a strain derived from USA011 by the Baltrus lab, isolated by plating out overnight cultures of USA011 on King's Medium B (KB) rifampicin agar plates and selecting a single colony. This single colony was then picked to KB media with rifampicin and frozen at -80°C in 40% glycerol. This frozen isolate was used to create DBL1424. DBL1424 is a deletion mutant derived from USA011R, in which the R-type syringacin receptor binding protein (Rbp) and chaperone genes have been deleted using a method originally described in Baltrus *et al.* 2012 (Baltrus *et al.* 2012). We have deleted both genes together for ease of complementation (they are at the 3' end of a predicted operon), but also because we have previously shown that these genes are exchanged together quite frequently (Baltrus *et. al* 2019). For more details on creation of this deletion, please see supplemental File S1 on Figshare. DBL1701 is a strain derived from DBL1424 in which deletion of the RBP and chaperone have been complemented and replaced *in cis,* please see supplemental File S1 for additional details about complementation and supplemental File S2 for Breseq results. These Breseq results demonstrate that the Rbp and chaperone have been deleted from USA011RΔRbp, complemented *in cis* in USA011RΔRbp+Rbp, and that the USA011RΔRbp+Rbp strain contains polymorphisms that demonstrate chain of lineage. Throughout the text, we refer to DBL1424 as "USA011RΔRbp" and DBL1701 as "USA011RΔRbp+Rbp".

Typically, for all experiments, *P. syringae* isolates were grown at 27°C on King's B (KB) agar and liquid media using rifampicin at 50 μg/ml. When necessary, cultures of both *P. syringae* and *Escherichia coli* were supplemented with antibiotics or sugars in the following concentrations: tetracycline at 10 μg/ml and 5% sucrose.

Sequencing and Assembly of Bacterial Genomes. The same protocol was followed for culturing of each strain prior to DNA extraction for sequencing. A single colony arising from original frozen stocks streaked to KB agar media was picked to 2mL KB broth and grown overnight at 27°C in a shaking incubator at 220rpm. Genomic DNA used for Illumina sequencing and Nanopore sequencing was isolated from these 2mL overnight cultures via the Promega (Madison, WI) Wizard kit with the manufacturer's protocols. RNAse A was added as per manufacturer's protocols for all of the genomic isolations.

Genomic DNA from USA011R was sequenced by the Baltrus lab via an Oxford Nanopore MinION using a R9.4 flowcell, with 1μg of DNA prepared using the LSK-109 kit without shearing. Reads were called during sequencing using Guppy version 3.2.6 using a MinIT (ont-minit-release 19.10.3) for processing. Sequencing on the MinION generated 35,408 reads for a total of 429,300,328bp of sequence with a read N50 of 22,807bp. Reads arising from Nanopore sequencing were used in conjunction with Illumina reads originally used to generate a draft sequence for this strain (Baltrus *et al.* 2014) using the hybrid assembler Unicycler (Wick *et al.* 2017) and with default parameters. Log files for the assembly can be found at File S4 on Figshare. This genome was annotated by NCBI's PGAP pipeline (Tatusova *et al.* 2016).

For USA011RΔRbp and USA011RΔRbp+Rbp, DNA was sequenced by MiGS (Pittsburgh, PA) using an Illumina platform following their standard workflow for library preparation and read trimming. As described in (Baym *et al.* 2015), this workflow uses a Illumina tagmentation kit for library generation, followed by sequencing on a NextSeq 550 with 150 base pair (bp) paired-end reads. Trimmomatic (Bolger *et al.* 2014) was used for adaptor trimming using the default settings. This workflow generated a total of 1,555,032 paired reads and 413Mbp (~68x coverage) of sequence for strain USA011RΔRbp and 1,368,760 paired reads and 368Mbp (~60x coverage) of sequence for strain USA011RΔRbp+Rbp. These reads were fed into the Breseq pipeline (Deatherage and Barrick 2014) and analyzed against the complete genome sequence of USA011R with default parameters to confirm genotypes for strains USA011RΔRbp and USA011RΔRbp+Rbp.

Tailocin Preparation and Quantification

Isolation of supernatants containing R-type syringacin molecules for strains USA011R, USA011RΔRbp, USA011RΔRbp+Rbp, *Psy*B728a, and *P. aeruginosa* PAO1 and *Psy*Cit7 were completed as outlined in steps one and two of (Hockett and Baltrus 2017). Briefly, bacterial cultures were grown on KB agar plates for 48 hours at 27°C. A single colony was picked to 3 mL KB media and grown overnight shaking at 27°C. The strains were then back diluted 1:100 and grown for 3-4 hours, then 2μL mitomycin C was added with a final concentration of 0.5 μg/ml in 3mL. The culture was then grown overnight, pelleted, and sterilized with chloroform the next day. Since the chloroform is separated and then evaporated, there is little to no chloroform in

142 the supernatant after this point. Supernatants can then be stored at 4°C until further use.
143 Tailocin preparations were not PEG precipitated for these experiments.
144 The quantification of the tailocins was completed for the experiments using strain
145 USA011R following the methods outlined in (Haag and Vidaver 1974; Mayr *et al.* 1972). Briefly,
146 tailocins were prepared as per (Hockett and Baltrus 2017) and diluted
147 1:10/1:100/1:10000/1:100000. 100 µL of these tailocin preparations were added to 900 µL samples
148 of an early log-phase culture of strain *Psy*B728a, and mixtures were placed on ice. Placement on
149 ice allows the tailocins to bind and lyse target cells, but largely prevents growth of the target
150 strain during this interval. After one hour, dilutions of each culture were plated on KB agar and
151 the number of viable colony forming units (CFUs) was determined for each mixture. Tailocin
152 concentrations were calculated by taking the CFU count from the lowest dilution of tailocin that
153 showed killing activity (N_k, for these datasets the dilution used was 1:100; Fig. S2B for strain
154 USA011 and Fig. S2C for strain USA011RΔRbp+Rbp), and comparing this number to the CFUs
155 from tailocin dilutions that showed no killing (N_0, for these datasets the dilution used was
156 1:10000; Fig. S2A). This comparison is therefore between a sample in which the tailocin has been
157 diluted so that it kills some (but not all) of the target cell population (N_k; $3x10^7$ for USA011 and
158 $1x10^7$ for USA011RΔRbp+Rbp) to a sample in which the tailocin is too dilute to have any effect
159 on overall number of CFUs (N_0, $4x10^8$). The tailocin concentration is then calculated as the
160 Poisson expectation (Mayr *et al.* 1972) from the comparison of these two numbers, back
161 calculated based on the dilution factor of the tailocins used in the calculations. Put differently,
162
163 Lethal units per µL = (ln (N_k / N_0)*(- N_0)) * (100, Dilution Factor) * (1/100 for assay dilution)

From these calculations, we estimate that there were ~1.0x10^9 tailocin particles per μL applied to plants during experiments with USA011 and ~1.4x10^9 tailocin particles per μL USA011RΔRbp+Rbp. Approximately 60 μL were added to plants during these treatments.

Plant Infections

Prior to infection experiments involving tailocins from *Psy*Cit7, individual seeds of *Nicotiana benthamiana* were placed into individual peat pellets (Jiffy, Lorain OH) and germinated and grown in the laboratory window using natural light. Plants were maintained in the window for 3-4 weeks in domed flats at which point they were used for infection experiments. Prior to infection involving tailocins from USA011R strains, *N. benthamiana* plants in domed flats were germinated and grown for 2-3 weeks in a growth chamber under 18L/6d scheme and at 65% humidity at which point they were moved to a laboratory window for infection experiments. We did not observe any large-scale or noticeable growth or disease differences in plants despite variable growth conditions mentioned above.

For infections, a small amount of *Psy*B728a was picked from a KB agar plate and grown overnight in KB media, pelleted and washed twice with 10mM $MgCl_2$, and then resuspended in an inoculation solution of 10mM $MgCl_2$ and silwet (40μL per 200mL) with bacteria at an OD600 of 0.05. For tailocin treatments, bacterial supernatants for each treatment were painted onto individual plant leaves using a sterile Qtip until the entire plant was covered. Tailocin applications within each treatment were performed at the same time using the same batch of tailocins for each experiment, with application of tailocins taking place on the order of minutes.

Approximately 60µL of tailocin was applied to each plant leaf, so that each leaf was coated in approximately $4x10^9$ tailocin particles. Plants treated with supernatants were left undomed for 1 hour, at which point they were dipped into the inoculum containing bacterial strain *Psy*B728a. Plants were then maintained in the laboratory window in domed flats until bacteria were sampled. At least 2 plants were infected for each treatment for each replicate (although the far majority of treatments consisted of 4 or greater plants per replicate), and each set of experiments consisted of a total of three replicates spread out over multiple weeks.

At 3 days post inoculation, the most diseased leaf from each plant was harvested into 500µL 10mM MgCl2. These infected leaves were macerated with small beads using a MP FastPrep-24 for 2 cycles at 20s per cycle. A dilution series from each of these samples was then plated out on KB media containing rifampicin and bacteria were enumerated after 3 days. We did not normalize for leaf size when enumerating bacterial populations. Since we plated 10µL from a 500uL of each sample, that the level of detection of these experiments is >50 viable cells per leaf.

Results

Application of tailocins from *P. syringae* strain Cit7 Protect *N. benthamiana* from infection by *Psy*B728a. Strain *Psy*Cit7 produces a tailocin that is active against *P. syringae* strain *Psy*B728a, while the tailocin produced by *Psy*B728a has no measurable activity against itself (Baltrus *et al.* 2019). Our *in planta* experiments also include additional negative controls which consist of preparations of R-type pyocins from *Pseudomonas aeruginosa,* which do not have measurable

activity against strain *Psy*B728a (Fig. S1 on Figshare), as well as a treatment with no supernatants applied to the plants. As one can see in Fig. 1, across 3 replicate trials, supernatant preparations from strain *Psy*Cit7 containing a tailocin against *Psy*B728a provide extensive protection against infection of *N. benthamina* if applied prophylactically before plants were inoculated with the pathogen compared to the no tailocin controls and the other treatments. This difference is statistically supported by a Kruskal-Wallis test ($\boldsymbol{X}^2$=34.812,df=3, p<0.0001) followed by pairwise Wilcox tests between each treatment (*Psy*Cit7 vs. No Tailocin, p<0.0001). These results stand in direct contrast to those found in the two other treatments (supernatants from *Psy*B728a vs. No tailocin, p=0.18) and *P. aeruginosa* PAO1 vs. No Tailocin p=0.53), which did not provide any additional protection from infection compared to the no tailocin control. Overall, the most striking result is that no cells of *Psy*B728a could be recovered by plating from plants treated with supernatants containing *Psy*Cit7 tailocins in 11/16 replicates.

Deletion of the Tailocin Receptor Binding Protein and Chaperone from USA011R Eliminates Killing Activity of Strain USA011R Against *Psy*B728a. In order to genetically test whether the production of active tailocins was required for protection of *N. benthamiana* from *Psy*B728a, we created a deletion mutant in which the tailocin receptor binding protein and chaperone were deleted from strain USA011R. We deleted both genes because we previously showed both that the dual deletion would disrupt tailocin activity (Baltrus *et al.* 2015) and also that these two genes appear to be transferred together as a group to modify tailocin activity in strains (Baltrus *et al.* 2019). We have previously shown that strain USA011R produces a tailocin that can specifically target *Psy*B728a (Baltrus *et al.* 2019). Whole genome sequencing of this strain

confirmed that deletion was created as intended in strain USA011RΔRbp (see Breseq results in File S2 on Figshare). We further sought to complement the deletion within strain USA011RΔRbp by replacing the deleted region *in cis* with DNA that nearly matched the original sequence from USA011R. Whole genome sequencing of USA011RΔRbp+Rbp confirmed that this region was successfully replaced with the exception that there is one silent single nucleotide polymorphism in the receptor binding protein compared to USA011R (see Breseq results in File S2 on Figshare). As shown in Fig. 2, deletion of the receptor binding protein and chaperone in strain USA011RΔRbp eliminates killing activity by this strain against *Psy*B728a. Furthermore, killing activity is restored when genes that encode production of these proteins are replaced *in cis* in strain USA011RΔRbp+Rbp.

Protection of *N. benthamiana* by *P. syringae* strain USA011R is Dependent on Functional Tailocin Production. In order to clearly demonstrate that active tailocins were the factor limiting infection of plants by *Psy*B7278a in our previous experiments, we repeated the tailocin protection assays using strain USA011R which possesses an R-type syringacin that is capable of targeting and killing *Psy*B728a. In this second set of trials, we included supernatants prepared from a mutant of strain USA011RΔRbp in which genes encoding the tailocin receptor binding protein and its chaperone were cleanly deleted. Lastly, we included supernatants from strain USA011RΔRbp+Rbp, in which the genes cleanly deleted from strain USA011RΔRbp were replaced *in cis* such that tailocin production was phenotypically complemented. As one can see in Figure 3A , preparations containing tailocins from strains USA011R and USA011RΔRbp+Rbp were able to protect *N. benthamiana* from infection by *Psy*B728a when applied as a prophylactic.

This difference is statistically supported by a Kruskal-Wallis test ($\boldsymbol{X}^2$=35.485,df=4, p<0.0001) followed by pairwise Wilcox tests between each treatment (USA011R vs USA011RΔRbp, p=0.00021; USA011RΔRbp+Rbp vs. USA011RΔRbp, p=0.00057). However, supernatant preparations from strain USA011RΔRbp showed no significant difference in the ability to protect plants from *Psy*B278a infection from either the *Psy*B728a supernatant preparation or the no tailocin treatment. Based on predictions from underlying genetics, supernatants from USA011RΔRbp should be identical to those from from USA011R and+Rbp except that the tailocin structures lack receptor binding proteins. As with *Psy*Cit7, no viable cells were recovered from a majority of plants treated with either USA011R supernatants (9/12) or USA011RΔRbp+Rbp supernatants (8/10).

As with the *Psy*Cit7 protection assays above, there was growth of strain *Psy*B728a during a limited number of replicates using supernatant preparations from either USA011R (3/12) or USA011RΔRbp+Rbp (2/10 infections). Again, in these 5/22 infections, disease symptoms were generally correlated with bacterial population sizes. As a follow up to investigate these escape situations, a single colony of strain *Psy*B728a was picked from the most diluted sample arising from each infected plant and tested for resistance to tailocins from USA011R. Every one of these tested colonies maintained tailocin sensitivity (Fig 3B).

We also note that the results reported above are clearly echoed in an experiment where plants were allowed to grow to 11dpi (Fig. 3C). As one can see in Fig. 3C, application of tailocins directed against *Psy*B728a to plants clearly protects plants from disease symptoms. However, plants that were untreated or which were treated with tailocins that do not target *Psy*B728a, show extensive disease (and death) at this later time point.

Discussion

Given their high target specificity and efficient killing capability, phage derived bacteriocins could provide important prophylactic treatments for agricultural crops against a suite of phytopathogens (Mills *et al.* 2017). Indeed, previous reports strongly suggested that application of tailocins from Pseudomonas could prevent infection of tomatoes by a *Xanthomonas* strain (Príncipe *et al.* 2018). As an added benefit, resistance mutations arising against tailocins have also been shown to severely affect the virulence of plant pathogens and may even sensitize strains to killing by alternative antimicrobials, so that the evolution of tailocin resistance under natural conditions may be inhibited by these tradeoffs (Kandel *et al.* 2020; Hockett *et al.* 2017). Lastly, since tailocins are non-replicating structures composed only of protein, it is possible that they could be more durable than phage treatments under the harsh conditions of agricultural fields and would not suffer from worries associated with uncontrolled release of phage (Meaden and Koskella 2013). Therefore, our goal with this manuscript was to extend these previously reported results to a new pathosystem and to establish a genetically controlled model system with which to begin to optimize tailocin application in which to see protective effects and which could be used to explore conditions to ensure efficient plant protection under a variety of conditions.

We demonstrate that tailocin production by two different strains (*Psy*Cit7 and USA011R) can effectively block infection of *N. benthamiana* by the phytopathogen *P. syringae* pv. *syringae* B728a (Figs. 1 and 3). These treatments proved highly effective, and in a far majority of cases

296 there were no cells of *Psy*B728a recovered from plants even though these plants were dipped in
297 inoculum containing 10^7 CFU/mL. In contrast, there was vigorous growth and infection of
298 plants by *Psy*B728a under control treatments where no supernatants were added to the plants
299 prior to infection. These results are especially clear at 11dpi (Figure 3B), where the tailocin
300 treated plants are grow quite vigorously while the plants without protection of tailocins are
301 dead or nearly so. Our experiments also demonstrate that supernatants prepared from
302 alternative strains, which produce tailocins that do not target *Psy*B728a, do not provide any
303 enhanced protection of plants than the no tailocin control.
304
305 Although our initial experiments were highly suggestive (Fig. 1), the possibility
306 remained that supernatants from strain *Psy*Cit7 contained additional (non-tailocin) molecules
307 compared to those from either *Psy*B728a or *P. aeruginosa* that could potentially protect plants
308 from infection or which could stimulate plant defenses prior to infection. To address this
309 critique, we generated a mutant strain of USA011R (lab strain DBL1424, referred to here
310 throughout as USA011RΔRbp) in which the tailocin receptor binding protein and its chaperone
311 were deleted from the genome as well as a strain (lab strain DBL1701, referred to here
312 throughout as USA011RΔRbp+Rbp) in which this deleted region was replaced with sequence
313 nearly identical to the region from strain USA011R. Deletion of these two genes in strain
314 USA011RΔRbp eliminates tailocin killing activity against strain *Psy*B728a, and this activity is
315 phenotypically complemented in strain USA011RΔRbp+Rbp (Fig. 2). Supernatants produced by
316 strain USA011RΔRbp are nearly identical to those produced by strain USA011R, except that the
317 tailocins in supernatants from strain USA011RΔRbp cannot bind to target cells and thus have no

killing activity. Experiments *in planta* using both of these strains (Fig. 3) clearly demonstrate that active tailocins are necessary to provide protection to plants against infection by strain *Psy*B728a.

In a small number of plants, tailocin treatments were ineffective in preventing infection by *Psy*B728a. We currently do not have an explanation for these results, but tested whether these infections were enabled through the evolution of genetic resistance against tailocins by isolating colonies arising from these infected plants and testing for tailocin resistance using overlay assays. In no case did we see genetic resistance against tailocins from USA011R when these colonies were restested. It remains a possibility that these rare instances of tailocin evasion were the product of persister-like phenotypes against tailocins which were recently reported (Kandel *et al.* 2020). Since it appears that the physiology of persister-like cells differs between broad-spectrum antibiotics and tailocins (Patel *et al.* 2021), it may be possible to generate effective combined treatments using either multiple tailocins or with combinations of different antimicrobial classes to eliminate all potential infectious cells. It could also be that a subset of cells periodically switches between tailocin resistance and sensitivity (perhaps through LPS modification (Simpson and Trent 2019)), and that this switch resets quickly enough to tailocin sensitivity when strains are grown under conditions for overlay experiments. In this case, developing "smart" treatments that include multiple tailocins against the same strain targeting different LPS moities or which include tailocins that generate collateral sensitive phenotypes could be effective (Hockett *et al.* 2017). Lastly, it may simply be that our crude application of tailocins to leaves was suboptimal in some cases and that with future experiments we could

optimize tailocin application to ensure complete protection. In this case, further experiments to optimize tailocin application may be useful for eliminating such escape infections.

Although we've demonstrated the ability of tailocins to serve as a source of prophylatic protection against infection, many bacterial diseases are not treated prophylactically for economic reasons, especially in vegetable crops. Future studies will assess whether application of tailocins is able to prevent spread of the pathogen within a field after a focal disease outbreak has occurred. It will also be of use to assess whether post-disease application of tailocins will be able to prevent formation of secondary infections on the same leaf. Lastly, we note that these and other studies point towards a possibility of engineering tailocins to be produced by plants as an additional layer of resistance against bacterial phytopathogens. However, the feasibility of the transgenic plant approach remains to be determined.

In sum, we report that application of phage derived bacteriocins to the leaves of *N. benthamiana* under the conditions described herein can reliably provide complete protection against infection by *P. syringae* strain B728a. These results support previous reports describing how application of tailocins to plants could prevent infection by phytopathogens. Our experiments expand on these previous reports by including a variety of different phenotypic and genetic controls which enable the clear attribution of causality to tailocins for these protective effects. We look forward to building on this system to optimize tailocin treatments to provide complete plant protection while also exploring the limits of tailocin protection of many different plant hosts against a wide range of phytopathogens.

Data Availability

Datasets underlying the results shown in Figures 1 and 3A, as well as the R commands used to create the figures, can be accessed at doi:10.5281/zenodo.5139181. All supplemental files and figures are found on Figshare at DOI: 10.6084/m9.figshare.12814205. A roadmap for creating deletion in strain USA011RΔRbp as well as for creating the complementation strain USA011RΔRbp+Rbp can be found at as File S1. Unmodified pictures used to create Figures 2 and 3B can be found as File S3A, File S5A, and File S5B. A complete genome sequence for strain USA011 is found at NCBI at accession GCA_000452525.4 , and raw sequencing read files used to assemble the genome of USA011R are found at SRR12282613 (Illumina) and SRR12282614 (Nanopore). Raw sequencing reads used to confirm genotypes of USA011RΔRbp and USA011RΔRbp+Rbp can be found in the SRA at SRR12516783 and SRR12516782 respectively.

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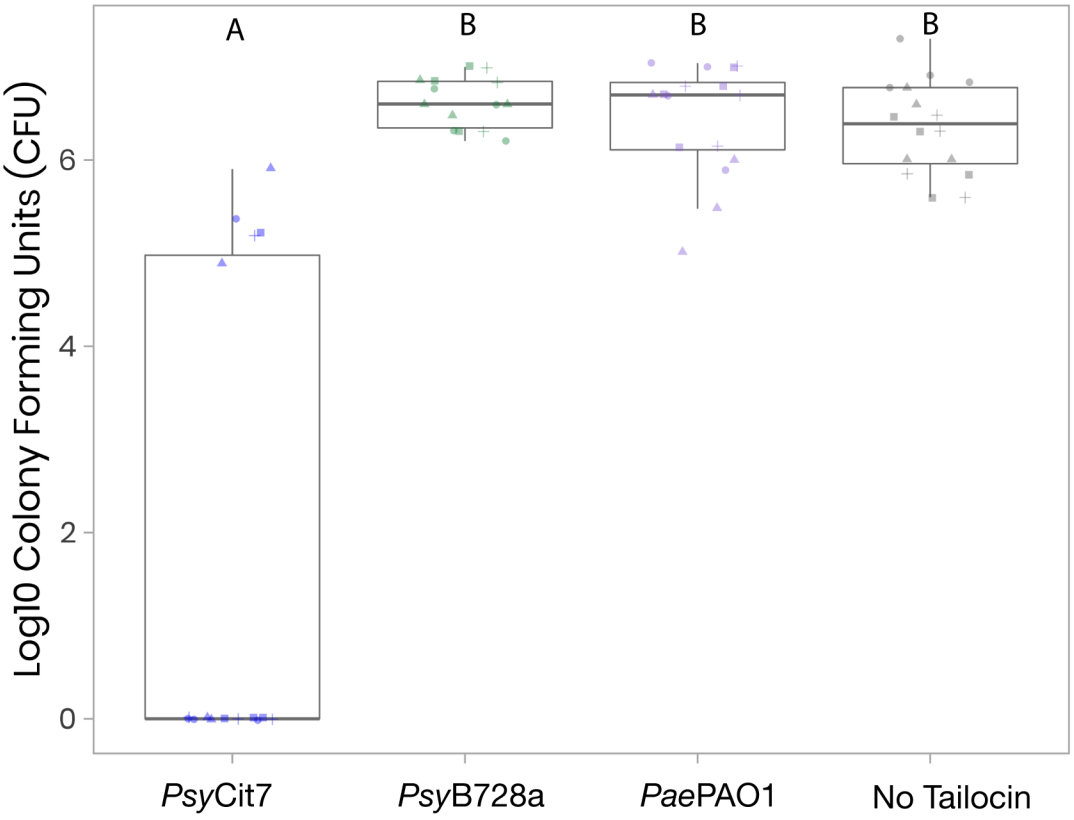


Figure 1: Supernatants Containing Tailocins from Strain *Psy*Cit7 Protect *N. benthamiana* from Infection by *P. syringae Psy*B728a. Shown on the Y-axis is the amount of strain *Psy*B728a recovered from an infected *N. benthamiana* leaf at 3 days post infection. Plants were pretreated with supernatants from a variety of strains (or no supernatant at all). Supernatants were produced by a variety of strains shown on the X-axis: *P. syringae Psy*Cit7 (which produces a tailocin that can target strain *Psy*B728a); *P. syringae Psy*B728a (which produces a tailocin but does not target itself); *P. aeruginosa* PA01 (which produces an R-type pyocin which does not target *Psy*B728a); and no supernatant applied. Data was gathered across three different experiments, shown by different shapes in the figure, with at least 2 (and most often 4)

391 replicates per experiment per strain. Groups that are significantly different ($p<0.01$) are
392 differentiated by letters according to results of pairwise Wilcox tests with correction for
393 multiple testing.
394

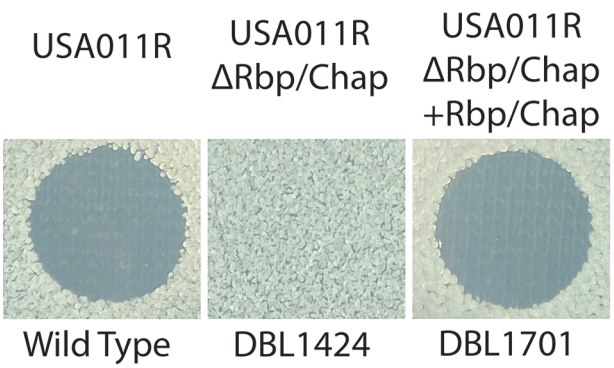
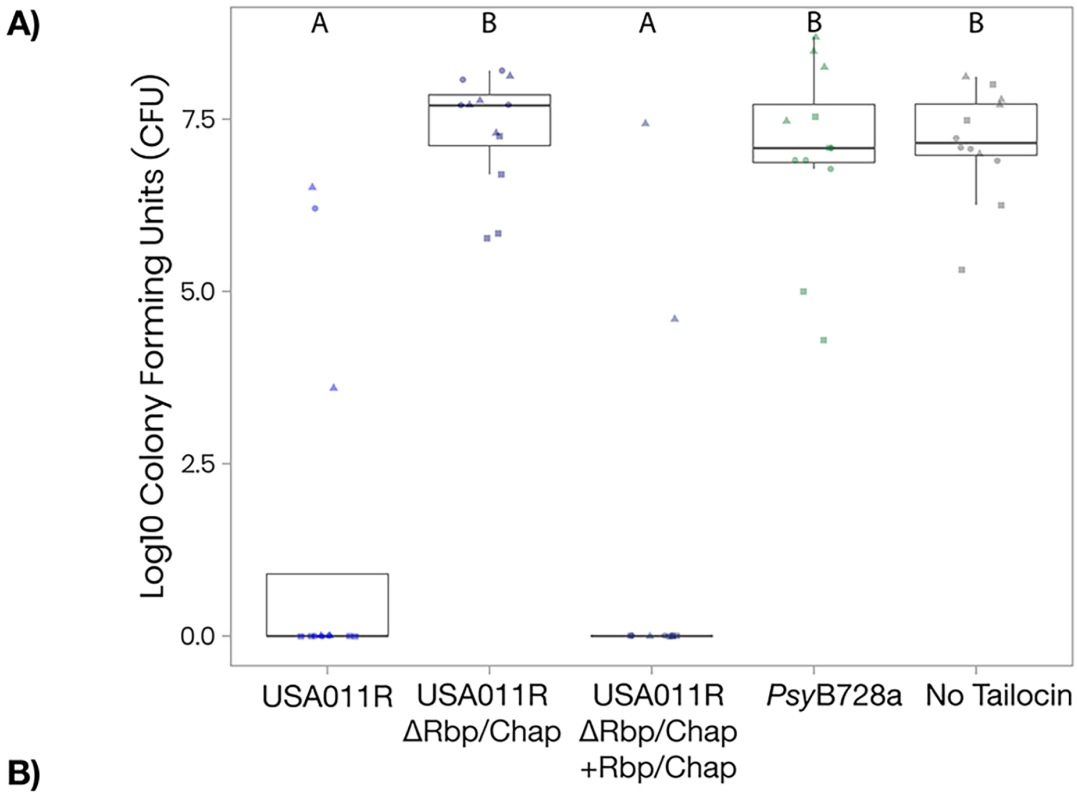


Figure 2: Deletion of the Receptor Binding Protein and Chaperone from Strain USA011R Eliminates Tailocin Killing of Strain *Psy*B728a. An overlay experiment, as per (Hockett and Baltrus 2017), was carried out in which tailocins were produced by strains USA011R, USA011RΔRbp, and USA011RΔRbp+Rbp. A clearing zone indicates that strain *Psy*B728a is killed by tailocins from both USA011R and USA011RΔRbp+Rbp, and a lack thereof demonstrates that this strain is not killed by tailocins from strain USA011RΔRbp


A)
A
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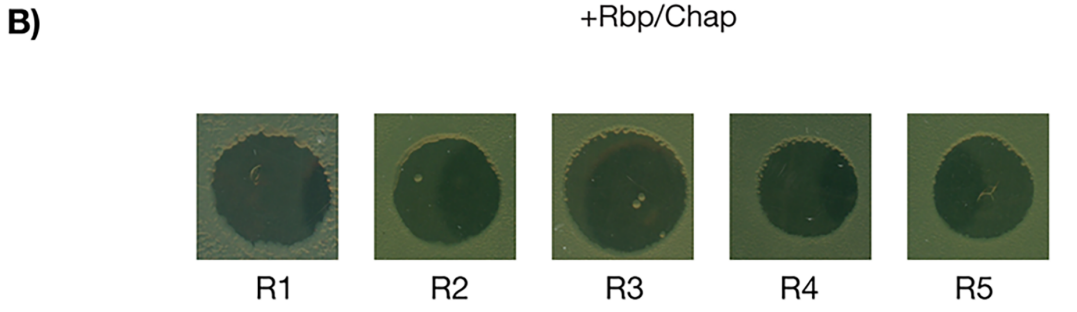
Log10 Colony Forming Units (CFU)
7.5
5.0
2.5
0.0
USA011R
USA011R ΔRbp/Chap
USA011R ΔRbp/Chap +Rbp/Chap
PsyB728a
No Tailocin


B)
R1
R2
R3
R4
R5


C)
USA011R
USA011R ΔRbp/Chap
USA011R ΔRbp/Chap +Rbp/Chap
PsyB728a
No Infection
No Tailocin

Figure 3: Tailocins from Strain USA011R Protect *N. benthamiana* from Infection by *P. syringae Psy*B728a. A) Shown on the Y-axis is the amount of strain *Psy*B728a recovered from an infected *N. benthamiana* leaf at 3 days post infection. Plants were pretreated with supernatants from a variety of strains (or no supernatant at all) derived from *P. syringae* USA011R, as shown on the X-axis. USA011RΔRbp is a deletion mutant in which the Receptor Binding Protein (Rbp) of the tailocin has been deleted. USA011RΔRbp+Rbp is a derivative of strain USA011RΔRbp in which the Rbp and chaperone from strain USA011R was replaced for complementation of strain USA011RΔRbp *in cis*. Data was gathered across three different experiments, shown by different shapes in the figure, with at least 2 (and most often 4) replicates per experiment per strain. Groups that are significantly different ($p<0.01$) are differentiated by letters according to results of pairwise Wilcox tests with correction for multiple testing. **B)** Single colonies were picked from plants infected despite application of preventative tailocins, and tested for sensitivity to these tailocins. All sampled colonies of strain *Psy*B728a arising from three plant infections after application of USA011R tailocins (R1, R2, R3) and both sampled colonies arising from two plant infections after USA011RΔRbp+Rbp tailocins (R4, R5) maintain sensitivity to killing by USA011R tailocins. **C)** An additional trial was carried out in which plants were pretreated with supernatants containing tailocins for the same strains as in A) except that this experiment also included a no infection control. In this experiment, plants were photographed at 11dpi and there were 4 replicate plants per treatment.

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