Evidence of Tailocin Persistence and Resistance in *Pseudomonas*

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Abstract

Tailocins are bacterially-produced, phage tail-like bacteriocins that have been proposed as therapeutics for treatment of bacterial infections. However, we have a limited understanding of how target populations survive tailocin exposure. In this paper, we demonstrate that cells of a target population of *Pseudomonas syringae* are able to survive lethal doses of a tailocin through both physiological and genetic mechanisms with stationary phase cells predominantly surviving using a physiological mechanism. Regardless of growth phase, a significant fraction of cells that survived tailocin exposure did not exhibit any increased resistance to subsequent exposure, indicating that these cells survive by persistence rather than resistance mechanism. Of those cells that did gain a detectable increase in tailocin resistance, there was a range from insensitive (complete resistance) to partially sensitive. We also recovered a mutant exhibiting a high-persistence like phenotype that showed significantly increased survival to transient exposure but no detectable growth after prolonged tailocin treatment. By sequencing the genome of multiple types of mutants, we identified several genes linked to lipopolysaccharide (LPS) biogenesis and implicated in tailocin tolerance and resistance. In particular, we found a specific genomic region that, when mutated, gave rise to the various classes of resistance and the high persistence phenotypes. Furthermore, we showed that a hypothetical gene involved in the high persistence phenotype is transcriptionally fused with the LPS biosynthetic region and contains a signal peptide and several trans-membrane domains. While the complete resistant mutants had lost their LPS O-antigen, incomplete resistant mutants contained minor to significant changes in their O-antigen. This work demonstrates that Gram-negative bacteria can survive tailocin exposure through multiple strategies, including the first description of a persistence-like mechanism for tolerating tailocin exposure.
Author Summary

With the rise in antibiotic resistant infections, it has become a necessity to study and find alternative treatment strategies. Bacteriocins, which are bacterially produced protein toxins, have been proposed as one of such alternatives. However, a deeper understanding of how target bacteria respond to bacteriocin exposure is lacking. Here, using a phage-tail like bacteriocin known as a tailocin, we show that target cells of the important plant pathogen *Pseudomonas syringae* are able to survive tailocin exposure through both physiological persistence and genetic resistance mechanisms. We show that bacterial cells that are not growing rapidly rely primarily on persistence mechanism, whereas rapidly growing cells are more likely to survive through genetic resistance. By genomic sequencing, we identified various types of mutations in the genes involved in lipopolysaccharide (LPS) biogenesis that cause increased tailocin persistence and resistance. In particular, we identified a mutation in a LPS-related genomic region that encodes a hypothetical protein and causes tailocin high persistence. We conclude that tailocin persistence is a real phenomenon and effects of both persistence and resistance in long term disease management needs to be considered before designing tailocins into biocontrol agents.

Introduction

The decreasing efficacy of commonly used antibiotics in treating bacterial infections of humans, animals, and crop plants is a significant concern for plant and animal health. According to a 2013 report of Centers for Disease Control and Prevention (CDC), in the United States alone, antibiotic resistant infections are estimated to cause two million infections and 23,000 deaths annually (1), with the number of deaths predicted to reach 10 million per year by 2050 (2). Although emergence of genetic resistance to antibiotics is a main driver of this trend, increasing
evidence suggests that non-heritable physiological persistence also plays a critical role in both antibiotic treatment failures and infection relapse (3-6). Resistance is a result of genetic changes that enable cells to withstand and grow in the presence of higher doses of an antibacterial agent, whereas persistence is an altered physiological response of a sub-population of genetically sensitive cells to survive transient lethal treatments (7). In addition to the altered physiological response that is mainly governed by gene expression changes, persistence can be caused by genetic mutations in genes associated to these physiological responses. Although resistance and persistence are considered distinct survival strategies, recent evidence suggests that physiological persistence can also reduce antibiotic efficacy by promoting the acquisition of resistance (8, 9).

As such, finding alternatives to treat bacterial infections is critical to combat the reduced efficacy of antibiotic therapies. More importantly, employing the alternative treatments in a manner that sustains their efficacy is critical for durable pathogen control. Bacteriocins, which are bacterially produced proteinaceous toxins, have been proposed as antibiotic alternatives (10). However, we currently lack a fundamental understanding of how bacterial populations are likely to survive bacteriocin treatments, should they be widely adopted.

Bacteriocins encompass diverse bacterial peptides, proteins, or protein complexes that are antagonistic mostly toward phylogenetically close relatives of the producer (11, 12). This potential of bacteriocins to inhibit the growth of competitor bacteria has been already utilized in food preservation to reduce the growth of food spoiling and pathogenic bacteria (13).

Bacteriocins are classified into different groups based on their structure, composition, and mode of action. Tailocins are bacteriocins that resemble bacteriophage tails and are grouped into R-type (with a retractile core tube) and F-type (flexible) (14, 15). Bacteria including the opportunistic human pathogen *P. aeruginosa* and other environmental Pseudomonads and
*Burkholderia* are known to produce tailocins that are believed to antagonize competitors including pathogenic strains among others (16-22). In fact, a recent study by Principe et al. showed the effectiveness of foliar sprays of tailocins produced by *P. fluorescens* in reducing the severity and incidence of bacterial-spot disease in tomato caused by *Xanthomonas vesicatoria* (22). Other studies have also indicated the potential use of engineered R-tailocins in suppressing food borne pathogens using *in vivo* models (23, 24). Our group has previously characterized a R-type tailocin from a plant pathogenic bacterium *P. syringae* pv. *syringae* (*Psy*) B728a (25). This R-type tailocin showed antagonistic potential against several pathovars of *P. syringae* that cause serious diseases and substantial losses in economically important crops such as common bean (pv. *phasiolicola*), soybean (pv. *glycinae*), chestnut (pv. *aesculi*), and kiwifruit (pv. *actinidae*) (25). A broad spectrum of tailocin mediated antagonistic interaction has been recently described in *P. syringae* (26).

In these systems, tailocins are considered to be potent killers as a single tailocin particle is predicted to kill a sensitive cell and an induced cell can release as many as 200 particles (27, 28). R-tailocins kill their targets by binding to the surface receptors and puncturing through the cell membrane causing dissipation of membrane potential and cell death (11, 27). Tailocins are known to recognize and bind specific lipopolysaccharides (LPS) components of the target cells to initiate killing (29-31). LPS is generally composed of three components: the lipid A, core oligosaccharide, and the O-polysaccharide (O-antigen) region (32, 33). The lipid A, and core region are mostly conserved within a species. However, the O-antigen region varies extensively in its chain length and composition of sugars (34). Biosynthesis and transport of LPS to the outer membrane as well the modification of O-antigen involves complex processes involving a number of highly diverse genes (33).
Although studies have demonstrated potent bactericidal activity of R-tailocins against pathogenic strains and have suggested their use in pathogen management (25, 26, 35), limited knowledge exists with regard to the efficacy of tailocins to provide sustainable pathogen management. Major limitations related to employing bacteriocins stem from our lack of understanding of how target pathogens might persist in the face of sustained treatment. Moreover, a detailed understanding of the genetics of LPS modification in relation to the evolution of tailocin resistance and persistence in the target pathogen is lacking. This knowledge is fundamental to assessing the sustainability of tailocins as therapeutic agents.

In this study, we aimed to determine the tolerance and resistance responses of target cells [Pseudomonas syringae pv. phaseolicola (Pph)] against tailocin produced by Psy. We exposed stationary and logarithmic cultures of Pph to lethal doses of tailocin and screened these surviving cells for resistant and tolerant phenotypes by tailocin re-treatment. We found that pronounced differences between the frequencies of resistant and persistent cells depending on growth phase of the target cells. Furthermore, we sequenced and analyzed the genomes of fourteen mutant lines exhibiting different levels of resistance and tolerance to identify mutations potentially contributing to each of these phenotypes. Of the heritable mutants, we identified ten unique mutant alleles with likely roles in the lipopolysaccharide (LPS) O-antigen biosynthesis leading to various degrees of tailocin resistance. We also identified an open reading frame (ORF) that is present within the operon containing LPS biogenesis genes and which encodes a hypothetical protein containing several transmembrane-domains and a signal peptide at its N-terminus. A mutation within this hypothetical protein led to increased tailocin persistence and stationary and log phase cells were no more different in tailocin tolerance for this mutant.
Materials and Method

Bacterial strains, media, and culture conditions

All bacterial strains and mutants used in this study are listed in Table 1. *P. syringae pv. syringae* (Psy) wild-type (WT) strain B728a and its tailocin defective mutant ΔRrbp were used to prepare the treatment supernatants. *P. syringae pv. phaseolicola* (Pph) 1448A was used as the target strain. Tailocin resistant and tolerant mutants of Pph generated in this study are described in Table 2. King’s medium B (KB) was used to culture the strains. Liquid cultures were prepared by inoculating individual colonies from a two-day old KB agar plate into 2 ml of liquid medium at 28°C with shaking at 200 rpm.

Tailocin induction and purification

Tailocin or control supernatants for treatment were prepared from logarithmic (log) cultures of *Psy* B728a and ΔRrbp, respectively using a polyethylene glycol (PEG) precipitation protocol as previously described (25, 36). Briefly, 100-fold diluted overnight B728a cultures were sub-cultured for 4-5 hours in KB before inducing with 0.5 µg ml⁻¹ final concentration of mitomycin C (GoldBio). Induced cultures were incubated for 24 hours with shaking at 28°C. Next, cells were pelleted by centrifugation and the supernatants were mixed with 10% (w/v) PEG 8000 (FisherScientific) and 1M NaCl. Supernatants were then incubated either in ice for 1 hour and centrifuged at 16,000 g for 30 min at 4°C or incubated overnight at 4°C and centrifuged at 7000g for one hour at 4°C. Pellets were resuspended (1/10- 1/20th of the original volume of the supernatant) in a buffer (10 mM Tris PH 7.0, and 10 mM MgSO4). Two extractions with equal
volume of chloroform were performed to remove residual PEG. Tailocin activity was confirmed by spotting dilutions of 3-5 µl of both the tailocin and control supernatants onto soft agar overlays of \( Pph \). The relative activity of tailocin was expressed as arbitrary units (AU) as obtained from the reciprocal of the highest dilution that exhibited visible tailocin killing in an overlay seeded with \( \sim 10^8 \) CFUs of \( Pph \) log cells. Lethal killing units of the purified tailocin were determined using a Poisson distribution of the number of surviving colonies after treatment with different dilutions of the tailocin as described previously (21, 37).

The relative activity of the purified tailocin was determined to be \( 10^3 - 10^4 \) AU and \( 1.25 \times 10^7 - 4.25 \times 10^9 \) lethal killing units/ml. The minimum inhibitory concentration was estimated to be 100 AU when exposed to \( \sim 10^6 \) viable target cells at their logarithmic growth. No loss of tailocin activity was observed for a period of over six months in the buffer (10 mM Tris PH 7.0, and 10 mM MgSO4) at 4°C.

**Tailocin treatment and survival assessment for stationary and log cultures**

To assess tailocin activity against the stationary and log phase of \( Pph \), individual colonies growing on KB agar plates for \( \sim 2 \) days were inoculated into 2 ml of liquid KB medium. Following incubation at 28°C with shaking at 200 rpm overnight, the cultures were back diluted 1000-fold into fresh KB. The back diluted cultures were either incubated for 28-30 hours to prepare stationary cultures, or back-diluted 100-fold at 24 hours and cultured for another 4-6 hours to prepare log cultures (see Fig S1 for a growth curve of \( Pph \)).

Stationary cultures were diluted 20,000-fold and logarithmic cultures were diluted 1,000-fold [\( \sim 10^5 - 10^6 \) CFUs/ml for both cultures see Fig 1] in fresh KB before tailocin treatment. Treatment was applied by mixing 10 µl of diluted cultures in 90 µl of purified tailocin preparation diluted in
After treatment, samples were incubated for ~1 hour at 28°C and washed twice to remove residual tailocin particles. Washing was performed by mixing the treated culture in 900 µl of fresh KB followed by centrifugation at 12,000 g for 2 min. The top 900 µl fraction was discarded and the bottom 100 µl fraction was serially diluted and either spread- or spot-plated to enumerate surviving population. Plates were incubated at 28°C for 2-3 days before enumeration. Serial dilutions of both stationary and log cultures were spotted onto KB agar to enumerate the untreated population. Experiments were performed with various tailocin concentrations (i.e. 100 AU, 500 AU, and 900 AU).

**Tailocin re-treatment to differentiate resistance and persistence**

Surviving colonies were treated again with tailocin to differentiate them into resistant or persistent colonies. Re-treatment was performed by an overlay method as described previously (36), or by broth treatment as discussed above. Overlay method was used to determine the AU of the tailocin preparation with the selected mutant lines. Broth exposure was used to calculate reduction in the population of log cultures. Surviving colonies were differentiated into various phenotypes as follows: persistent (sensitive to tailocin to the wild-type level in both the broth and overlay method), high-persistent like (completely sensitive in the overlay but survived significantly more than wild-type under broth condition), incomplete resistant (showed some level of sensitivity at least in one of the treatment conditions), and complete resistant (were insensitive under both conditions).

**Time dependent death curve with tailocin treatment**

Prolonged tailocin exposure was performed with both the stationary and log-phase cultures of *Pph* to generate death curves. Treatment was applied in a 96-well plate as before. Surviving
populations were enumerated at 1, 4, 8, and 24 hours following treatment and randomly selected surviving colonies were re-exposed to tailocin to differentiate them into persistent or resistant phenotypes.

**Tailocin recovery from the treated samples and activity testing**

Stationary and log cultures treated with tailocin for 1-24 hours as described above were centrifuged and the supernatant was collected, and filter sterilized using a 0.22 µm syringe filter. Supernatants were diluted 5-, 10-, 50-, and 100-fold in KB and spotted on *Pph* overlay. Purified tailocin particles diluted in KB were also included as control treatment.

**Determining the effect of stationary and log supernatant on tailocin activity**

Stationary and log phase cultures were prepared as described above by culturing *Pph* cells in KB broth for either 28-30 hours or for 4-6 hours, respectively. Cultures were centrifuged for 2 min at 12,000 g and the supernatant was filter sterilized using a 0.2 µm syringe filter. Stationary and log supernatants were diluted 1,000-20,000-fold in KB (according to how the cultures were diluted for tailocin treatment). Various dilutions (10-, 50-, 100-, and 1000-folds) of purified tailocin were prepared in the stationary and log supernatants. Dilutions were spotted on a *Pph* lawn using the overlay method. AU of the of the tailocin suspensions were calculated as described above.

**LPS extraction and visualization**

LPS extraction was performed as described by Davis and Goldberg (38) from pellets of one milliliter of overnight cultures (OD$_{600}$ 0.5). After extraction, 10 µl samples were separated by SDS-PAGE. Samples were labeled using the Pro-Q Emerald 300 glycoprotein stain kit (ThermoFisher Scientific, #P21857) according to manufacturer's instructions. For LPS size
determination, CandyCane™ glycoprotein molecular weight standards (ThermoFisher Scientific, #C21852) were included. Gels were visualized using Molecular Imager Gel-Doc XR+ (Bio-Rad) with Image Lab Software.

**Genome sequencing and analysis of the tailocin high-persistent and resistant mutants**

The tailocin high-persistent like (HPL) and complete and incomplete resistant mutants recovered from tailocin treatment of *Pph* wild-type cells (and confirmed by re-treatment) were selected for genome sequencing. DNA was extracted from the overnight cultures using Promega Wizard Genomic DNA purification Kit using manufacturer’s protocol. DNA quantity and quality were assessed with Qubit 3 Fluorometer using Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific) and Nanodrop 2000 (Thermo Scientific). A uniquely indexed library from each mutant line was prepared using Illumina DNA Flex kit (Illumina). An approximately equimolar pool of libraries was generated and 150bp paired-end reads were sequenced on an Illumina MiSeq.

Resulting forward and reverse read files were paired and mapped to the *Pph* 1448A chromosomal and plasmid sequences using Geneious R10.2 with default parameters for medium sensitivity. Next, genetic variants were identified using ‘Find variations/SNPs’ program within Geneious using default settings. Regions supported by a minimum of 10 reads and >90% variant frequency were selected. Moreover, variants shared among all the mutants that were generated in independent experiments were discarded as misalignments. Variants identified by Geneious were confirmed in the contigs generated by De Novo assembly of the paired reads using SPAdes 3.11.0 using K-mer sizes of 21, 33, 55, 77, 99, 127 with careful mode selected to minimize mismatches and short indels. Indels were also detected and visualized in the contigs by Harvest.
suit tools (39). Sequencing generated 1,986,400 -2,857,324 paired reads per genome with 48-69X genome coverage. *De novo* assembly generated 308-338 contigs per genome with the N50s of 75,222-81,220bp. The total assembly size was 5.97 Mbp with a GC content of 57.96%, values similar to *Pph* reference genome (40).

Presence absence and homology searches of the selected genomic regions and genes implicated in tailocin resistance and tolerance were performed with NCBI and IMG-JGI databases using BLAST algorithm using the *Pph* sequences as query. InterProScan (41) and Phobious program within the Geneious plugin was used to predict functional domains in the amino acid sequences.

**Statistical analysis**

Means of total and surviving population between treatments were compared using the Glimmix protocol in SAS 9.4 with experimental repeat used as a random factor. Whenever required, post hoc analysis was performed with Tukey’s Honest Significant Difference (Tukey HSD) test at 5% significance level ($P=0.05$).

**Results**

**Tailocin persistence increased in the stationary state**

Purified tailocin supernatant was used to test its killing effects on stationary and log phase cultures of the *Pph* target cells in a broth environment. After an hour of 100 AU tailocin treatment, a consistent reduction ($3.59 \pm 0.12$ log reduction) in the viable population occurred for logarithmic cultures, while a significantly lower reduction ($1.38 \pm 0.14$ log reduction) occurred for the stationary cultures. Further analysis showed that, upon treatment of equivalent number of viable cells, stationary cells consistently survived 10 to 100-fold more than the logarithmic cells.
Surviving colonies, especially those from the stationary phase, were predominantly sensitive upon tailocin re-exposure suggesting survival by persistence mechanism (see below).

A persistent sub-population was maintained under prolonged exposure time and increased concentration of tailocin

Tailocin treatments were applied to both stationary and log cultures of Pph for up to 24 hours with enumeration of surviving population before and after 1, 4, 8, and 24 hours of tailocin treatment to generate a tailocin death curve. After a steep reduction in the population within the first hour of tailocin treatment, further killing of the cells that survived the first hour treatment, did not occur in either culture (Fig 2A). Twenty-four hours post-treatment, although the overall population increased (Fig 2A), individual treatments showed different results: for some replicate treatments the population remained constant suggesting maintenance of the persistence state, while for some other replicates, population increased due to division of cells that acquired tailocin resistance (see Fig S3).

Upon tailocin re-treatment, >90% of stationary and >60% of log cells that survived the first hour treatment, were as sensitive as the WT (i.e. persistent) as in (Fig 2B). The proportion of persistent survivors was higher in the stationary cultures compared to the log cultures at all time points (Fig 2B). Tailocin persistent cells were recovered from both cultures even after 24 hours of tailocin treatment, although the proportion decreased over time (Fig 2A). Tailocin activity (see Fig S4) was detected in the supernatants recovered from the treated samples that contained tolerant cells, confirming saturation of tailocin in the treatment. Although a slight reduction of activity was observed when the tailocin preparation was mixed with undiluted stationary
supertant compared to log supertanant, no difference was detected upon diluting the supertants up to 1,000-20,000-fold (as the cultures were diluted for tailocin treatment) before mixing with tailocin (Fig S5). This suggested that the increased tailocin tolerance in the stationary phase is not related to inhibition of tailocin activity by an extracellular component.

Upon treating the cells with a concentrated tailocin (900 AU) the surviving population decreased such that no difference in survival between the stationary and log cultures was detected (Fig 3A). However, even with this higher level of tailocin applied, the proportion of tailocin persistent cells remained higher for stationary phase survivors than that for the log phase survivors (Fig 3B).

Tailocin exposure selected for heritable mutants showing heterogeneous resistance and increased persistence

In addition to the recovery of tailocin persistent sub-population, we recovered complete tailocin resistant mutants (i.e. heritable mutants that are insensitive to lethal doses of tailocin), and incomplete resistant (IR) mutants (see Fig. 2B and 3B for proportion) that were still sensitive to tailocin, but with sensitivity significantly decreased compared to the wild-type cells both in liquid-broth and agar-overlay conditions (Fig 4A and 4B). Moreover, we recovered an unique phenotype, refered here as high persistent-like (HPL), that, compared to the wild-type, showed increased persistence in liquid-broth treatment but equivalent sensitivity in an agar overlay setting (Fig 4A and 4B). Both the IR and HPL phenotypes were heritable as progeny colonies carried the same phenotype. Furthermore, the HPL phenotype did not differ in survival between the stationary and log phase (Fig 5).

LPS analysis of the mutants and the wild-type Pph showed that the complete resistant mutants lacked fully-formed O-antigens, whereas the high-persistent and majority of incomplete resistant
mutants still possessed the O-antigen with subtle changes. One of the incomplete resistant mutants (IR4), however, showed a very different and faint O-antigen band (Fig S6).

Mutants showed modification of genes of lipopolysaccharide biogenesis by single-nucleotide polymorphisms and mobilization of miniature inverted-repeat transposable element (MITE)

Genome sequencing and variant identification of the high-tolerant like (n=1), incomplete resistant (n=6), and resistant mutants (n=4) was performed by mapping the Illumina reads with the parental Pph 1448A reference sequence. Mutations were detected in several potential genes responsible for lipopolysaccharide O-antigen biosynthesis in the complete and incomplete resistant mutants (Table 2). Mutants isolated at different experiments showed mutation in a different gene. A specific region (Fig 6) was identified in the Pph genome that showed the most prominent role in tailocin tolerance and resistance. Even within a single gene (PSPPH_0957), different phenotypes were identified depending on the nature of the mutation (see Table 2 and Fig 6). Moreover, one of the incomplete resistant mutant class (IR6) showed mobilization of the 100 bp MITE sequence present in the Pph genome as described by Bardaji et al (42). The insertion of this MITE inactivated the target gene (PSPPH_0963) causing the incomplete resistance phenotype. The high tolerant mutant had a 16 bp deletion that caused frameshift in the ORF of a transmembrane domain containing hypothetical protein.

Bioinformatics of mutated genes
Genes identified to have a role in tailocin activity (see Table 2 for a list) were assessed for their presence and similarity to those found in other Pseudomonas genomes. PSPPH_0957 is predicted to encode a glycosyl transferase (family 1) and conferred both complete and incomplete resistance phenotypes depending on the type of mutation (see Fig 6) and is present across majority of sequenced P. syringae isolates. However, the region from PSPPH_0958 to PSPPH_0964 displays much more variation within P. syringae. For instance, a pv. glycinea race 4 isolate, which is closely related to Pph, lacks this region. Although predicted orthologs for some of the genes in this region were present, PSPPH_0963, and PSPPH_0964 that occur contiguously in an operon were less abundant even within P. syringae (present only in 87 genomes out of 287 P. syringae genomes in IMG-JGI database). Orthologs of these two genes were not found in the genomes of pv. tomato (DC3000), Psy (B728a), and Pgy (R4), but were found in strains of pv. actinidiae, pv. morsprunorum, pv. aesculi and some environmental isolates. Although the incomplete resistance gene (PSPPH_0963) was identified to have a FAD/NAD(P) binding cytoplasmic domain with a potential role in LPS biogenesis (IMG product name UDP-galactopyranose mutase), no functional role could be predicted for the hypothetical gene involved in the high-persistent like phenotype. Nevertheless, it possesses ten predicted transmembrane domains and a signal peptide domain at its N-terminus.

Discussion

There has been a renewed research interests in alternative treatment strategies for bacterial pathogens due mainly to the growing threats of antibiotic resistant infections. Tailocins have long been proposed as effective and more specific alternatives to broad spectrum antibiotics. However, the evolutionary dynamics of tailocin persistence and resistance have not been widely investigated. In this study, we addressed this fundamental concern using a phage-tail like
bacteriocin (i.e. tailocin) produced by *P. syringae* pv. *syringae* strain B728a in killing target cells of *P. syringae* pv. *phaseolicola* 1448A. Upon exposure of a lethal dose of tailocin to equal numbers of stationary and logarithmic *Pph* cells, a higher fraction of stationary cells survived than cells in log phase. Upon re-exposing surviving cells, a majority of the cells were sensitive to the wild-type level, suggesting that non-heritable persistence rather than heritable resistance was the predominant survival mechanism, particularly in the stationary phase. A prolonged tailocin exposure generated a killing pattern similar to the one reported for persistent sub-population upon antibiotic treatment (7, 43). Persistence was maintained for at least 24 hours with tailocin exposure, a phenomenon that was more evident in some experimental replicates in which resistance evolution was not observed. However, by increasing the tailocin concentration in the treatment, we showed that most of the persistent survivors were killed, and the difference in survival between the two growth phases was no longer seen. Upon re-exposure, however, stationary phase-derived cells exhibited a higher fraction of persistence. Overall, these experiments using various tailocin concentration and exposure time suggested that tailocin persistence is dependent on concentration rather than duration of exposure. We showed that increasing the concentration of the tailocin treatment enabled killing of even the highly persistent stationary cells. This indicated that the stationary cells may require multiple hits by tailocin particles as opposed to the one-hit-one-kill mechanism of killing described for tailocins (27, 28), or that the probability of a successful hit in stationary phase is lower than in log phase. Since tailocins are specific to their targets, and are not known to have off-target effects, higher concentration of tailocin could be used to achieve a more effective pathogen control. However, although at a low level, persistence was still maintained even with high-dose tailocin treatment and inherent emergence of either complete or incomplete resistance was frequently observed. As
such, although a significant reduction in pathogen population and disease pressure can be
obtained with tailocins, a stand-alone tailocin treatment might not be enough to achieve a
sustainable pathogen control.

The use of the term ‘persistence’ in relation to antimicrobial survival is disputed to some extent
and is sometimes used interchangeably with ‘tolerance’ and ‘viable but not culturable state’. In
this paper, we used the term ‘persistence’ as this trait was only seen in a sub-population, resulted
in a bi-phasic death curve, the surviving persister cells resuscitated in the absence of the tailocin
treatment, and were equally sensitive to the wild-type cells upon re-exposure. This definition of
persistence has been suggested previously (7). Persistence to antimicrobials is being increasingly
recognized for its role in antimicrobial treatment failures with bacterial infections (44). Various
mechanisms such as formation of persister-subpopulation or biofilms are implicated in the
maintenance of persistence (45, 46). However, persistence responses can be different based on
the stresses involved and their mode of action as reported previously with different antibiotic
treatments (47). Several toxin-antitoxin (TA) systems are implicated in the formation of
persister-subpopulation (4, 6, 48). TA systems were shown to be induced when cells were
starved for certain sugars and amino acids or by exposure to osmotic stresses that altered ATP
levels in the cell (47, 49). However, TA system activation did not always induce persister
formation (49). Additionally, recent findings have indicated a mechanism mediated by the
guanosine pentaphosphate/tetraphosphate (ppGpp) for persister formation that is not dependent
on a TA system (50). Instead, a strong stationary state effect, that likely involved starvation
response, was shown to increase persistence by 100-1,000 fold in Staphylococcus aureus with
ciprofloxacin treatment (49). Whether similar mechanisms of TA and/or ppGpp systems regulate
tailocin persistence or a specific mechanism for tailocin and/or related bacteriophage exists,
remains to be determined. Nevertheless, our data of the difference in tailocin tolerance between the stationary and log cultures suggests that metabolic inactivity and starvation induced stress could be a strong factor in tailocin persistence.

Few previous studies have demonstrated growth-phase dependent differences in LPS O-antigen chain length and composition or their regulatory pathways (51, 52). Furthermore, in a previous study with *P. fluorescens*, exponentially growing cells had a significantly higher rate of cell lysis than stationary or decline phase cells with bacteriophage PhiS1 (53). It could be possible that the stationary phase cells possess an altered O-antigen that interferes with tailocin binding and target recognition, causing the difference in tailocin survival between the two growth phases. This could be supported by our observation that un-diluted stationary phase supernatant inhibited tailocin activity to some extent compared to the log phase supernatant. Moreover, the finding that mutation in one of the hypothetical proteins containing a signal peptide and several transmembrane domains caused increased tailocin persistence is noteworthy. More importantly, the stationary and log state difference in tailocin survival was lost for this mutant. Since the hypothetical protein occurs in the same operon as other LPS biogenesis genes, it is likely that it plays a role in O-antigen biogenesis and maintenance, thereby reducing tailocin interaction with the cells.

Another mechanism that can cause increased survival to surface active antimicrobials (eg. phages and host immune defenses) is phase variation (54). Phase variation is a gene regulation system that induces heterogenous expression of specific genes in a clonal population (54-56). Phase variation is heritable but reversible as the cells go through generations. The ‘ON’ ‘OFF’ switch occurs randomly amounting to $10^{-4}$ to $10^{-1}$ per generation, significantly greater than is expected by mutational events (57). Phase variation has been shown to modify LPS operons in *S.*
enterica spp (58). Moreover, temporal development of phage resistance in S. enterica serovar Typhimurium by phase variable glucosylation of the O-antigen has been evidenced (59). In addition, phase variation in P. fluorescense has been demonstrated during rhizosphere colonization of Alfala. The phase variants were enhanced in motility by production of a longer flagella than the wild type cells and were able to colonize distal end of the roots (60). It can be expected that phase variation could be a part of tailocin survival mechanism, particularly because of the fact that LPS, that is commonly modified in bateria by phase variation, serves as the receptor for tailocins. Previous studies with treatments of bacteriophage that recognize the target cell LPS have evidenced the role of phase variation in phage defense (61, 62), including in the closely related P. aeruginosa (63). However, whether tailocin persistence observed here is the result of phase variable LPS genes, needs further assessment. Nevertheless, in one of the incomplete resistant mutants, the target gene was modified by movement and integration of an internal mobile genetic element (MGE). MGEs have been described to have a role in causing phase variation in other bacterial systems (64, 65). Further hightthroughput screening techniques that can test the sensitivities of thousands of progeny CFUS of the incomplete resistant mutants will be required to determine the rate of switch to confirm if this is a case of phase variation.

To our knowledge, the incomplete resistant phenotypes observed here have not been described before with bacteriocins although few studies have discussed bacteriocin persistence (66, 67). Here, using a phage-tail like bacteriocin, we showed that resistant lines with various degrees of sensitivity are selected by exposure of target cells to tailocins. As the complete phage resistant mutants have been shown to be defective in virulence and fitness (68), incomplete resistance, which likely has a less pronounced fitness cost, might be a better survival strategy. In the incomplete resistant mutants, the LPS O-antigen region was present, but modified at a gross
level, unlike in the complete resistant mutants that had completely lost their O-antigen.

Preserving the O-antigen and still surviving the tailocin or phage attack may prevent the fitness cost associated with complete resistance evolution. Further tests that will compare the virulence and fitness traits of the incomplete resistant mutants together with the wild-type and resistant lines will be required to confirm these hypotheses.

The LPS region of *Pph* containing genes involved in incomplete resistance and tolerance was not found in some other isolates of *P. syringae*. For instance, pathovar *glycinea* (*Pgy*) race 4, that is very closely related to *Pph* (69, 70) and occurs within the same genospecies (genospecies 2) and phylogroup (phylogroup 3) based genome-genome hybridization and multi-locus sequence typing of housekeeping genes (71, 72), respectively, lacked this genomic region and the homologs of some of the genes occurring in this region. However, even though *Pgy* race 4 lacks this LPS region, this strain was shown to be tailocin sensitive in previous studies (25, 26). This supports our observation that mutation of this region does not completely eliminate tailocin sensitivity, but gives rise to various incomplete resistant and tolerant phenotypes.

Although LPS O-antigens are known to be tailocin receptors, the involvement of specific genes and LPS regions in tailocin recognition has not been clarified in *P. syringae*. In this study, we identified several genes of LPS O-antigen biosynthesis, and showed that mutations of some of these genes causes complete loss of O-antigen and therefore complete tailocin resistant mutants. On the other hand, other mutations at the same or different genes cause potential change in the O-antigen chain length and composition causing incomplete resistance. Some complete resistant mutants showed a typical rough-colony morphotype due to lack of O-antigen. Rough-colony morphotypes lacking O-antigen were resistant to phages as described previously in *P. syringae* pv. *morsprunorum* isolates from plum and cherry (73). Moreover, these rough colony
morphotypes showed reduced virulence in the plants (73). In other plant pathogenic bacteria such as *Xanthomonas oryzae* pv. *oryzae* and *Xylella fastidiosa*, loss of O-antigen, respectively, reduced type III secretion into plants (74), and increased recognition of the pathogen by the host immune response (75). In both cases, plant virulence of the mutants was significantly reduced (74, 76). This suggests that the complete tailocin resistant mutants that lack the fully-formed O-antigen might, in fact, lose plant fitness. Other means of survival such as tolerance, and incomplete resistance described here might therefore serve to counter the fitness cost associated with gaining complete tailocin resistance, enabling survival from the attack of both the co-infecting competitors as well as host defense responses.

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Table 1. Bacterial strains and mutants used in this study

| Strain        | Background                          | Characteristics                  | Source |
|---------------|-------------------------------------|-----------------------------------|--------|
| B728a         | *P. syringae pv. syringae* (Psy)    | Tailocin producing WT strain     | (77)   |
| B728a:ΔRrbp   | *Psy*                              | A tailocin deficient B728a mutant | (25)   |
| 1448a         | *P. syringae pv. syringae* (Pph)   | A tailocin sensitive WT strain   | (40)   |

Table 2. Predicted mutation and genomic changes in tailocin tolerant and resistant mutant

| Mutant        | Log_reduction | Locus       | Gene                                     | Change                          | Gene abundance across *P. syringae* isolates |
|---------------|---------------|-------------|------------------------------------------|---------------------------------|---------------------------------------------|
| *Pph*         | 4.1±0.219     | wt          | -                                        | -                               | Low                                         |
| HPL           | 2.95±0.36     | PSPPH_0964  | Hypothetical protein                     | 16 bp deletion, frameshift     | Low                                         |
| IR1           | 0.48±0.08     | PSPPH_3226  | Glycosyl transferase                     | T>P                             | High                                        |
| (BRM279)      |               |             |                                          |                                 |                                             |
| IR2           | 0.78±0.28     | PSPPH_2810  | 4-amino-4-deoxy-L-arabinose transferase  | S>I                             | High                                        |
| (BRM233)      |               |             |                                          |                                 |                                             |
| IR3           | 0.33±0.14     | PSPPH_0960  | Glycosyl transferase family 2            | (N>I)                           | High                                        |
| (I3)          |               |             |                                          |                                 |                                             |
| IR4           | -0.10±0.05    | PSPPH_0957  | Glycosyl transferase                     | T insertion, extra AA added at the end | High                                        |
| (BRM11)       |               |             |                                          |                                 |                                             |
| IR5           | -0.1±0.07     | PSPPH_0520  | Glycosyl transferase                     | H>R                             | High                                        |
| (BRM334)      |               |             |                                          |                                 |                                             |
| IR6           | -0.260±0.06   | PSPPH_0963  | FAD-dependent oxidoreductase             | 100 bp insertion,              | Low                                         |
| (I6)          |               |             |                                          |                                 |                                             |
|  |  |  |  |  |
|---|---|---|---|---|
| R1 | -0.26±0.06 | PSPPH_0957 | Glycosyl transferase | truncated protein (mite) C>A substitution, truncated protein |
| (BRM16) | | | | High |
| R2 | -0.17±0.08 | PSPPH_0957 | Glycosyl transferase | E>K |
| (BRM15) | | | | High |
| R3 | -0.19±0.15 | PSPPH_0952 | Nucleoside-diphosphate-sugar epimerase | R>W |
| (BRM258) | | | | High |
| R4 | -0.22±0.17 | PSPPH_0983 | Glycosyl transferase | Frameshift |
| (BRM328) | | | | High |

**Figure legends**

**Fig 1. Difference in tailocin survival between stationary and log phase cultures of Pph.**
Cultures were treated with a lethal dose of tailocin and viable cells pre- and post-treatment were enumerated. Experiments were repeated at least five times with 3-6 biological replicates per time. Mean and standard error of mean are graphed. P<0.05 indicate significant differences within grouped bars as analyzed in SAS 9.4 with proc Glimmix.

**Fig 2. Time dependent dynamics of tailocin survival. A) Stationary and log cultures of Pph were treated with tailocin and the viable population was enumerated before and 1, 4, 8, and 24 hours post-treatment. Three independent experiments were performed with 3-6 biological replicates per time. Mean and standard error of mean are graphed. Different letters represent significant differences as analyzed in SAS 9.4 with proc Glimmix at P=0.05. B) Percentage of surviving colony phenotype upon tailocin re-treatment. Randomly selected surviving colonies
(n=12-44 for each growth phase and hours of treatment) from the initial tailocin treatment were
sub-cultured and re-treated with tailocin, and percentage of the surviving phenotype was
calculated. Colonies recovered from three independent experiments were used for the re-
treatment to calculate this percentage.

**Fig 3. Dynamics of tailocin survival with concentrated tailocin treatment.** A) Cultures were
treated with high dose of tailocin (900 AU) and viable populations pre- and post-treatment were
determined. Experiments were repeated at least three times with 3-6 biological replicates per
time. Mean and standard error of mean are graphed. \( P<0.05 \) indicates significant difference
within grouped bars as analyzed in SAS 9.4 with proc Glimmix. B) Percentage of surviving
colony phenotype after treatment with concentrated (900 AU) tailocin for one hour. Although
most of the surviving colonies were either incomplete resistant or resistant, tolerant cells
persisted even at high tailocin concentration. Surviving colonies were tested during three
independently repeated experiments for both cultures.

**Fig 4. Treatment response of tailocin persistent and resistant lines.** A) Reduction in the
population of tailocin persistent and resistant mutant lines upon re-treatment with tailocin. Log
cultures of each lines were treated with 900 AU of tailocin and change in the population was
calculated after an hour of tailocin treatment. At least three separate colonies of each lines were
tested and experiments were repeated a minimum of three times. Means of the difference in log
transformed viable population pre- and post treatment are graphed. Error bars indicate standard
error of mean. B) Assessment of response of mutant lines to tailocin under overlay condition.
Dilutions of tailocins (shown on the left most column) were spotted over the culture lawn of each
of the lines. Yellow line indicates the dilution up to which visible killing was observed. HPL;
high persistent-like, IR; incomplete resistant, R; resistant.
Fig 5. Dynamics of tailocin survival for the high persistent-like (HPL) mutant. Cultures were treated with various tailocin doses and viable cells pre- and post-treatment were enumerated. Means and standard error from three independently repeated experiments with at least three biological replicates per experiment are reported. More killing occurred at high tailocin concentration and no difference in stationary and log phage was observed. $P>0.05$ indicate no significant differences within grouped bars as analyzed in SAS 9.4 with proc Glimmix.

Fig 6. Mapping a genomic region (PSPPH_0957- PSPPH_0964) of Pph that showed prominent effect on tailocin sensitivity and resistance. Mutations of this region gave rise to multiple phenotypes that differed in tailocin tolerance/resistance. Refer Table 2 for a complete list of genes at this and other genomic locations.
Before

After 1 hour (100AU tailocin)

$P = 0.9$

$P < 0.001$
900 AU tailocin applied to log cultures for 1 hour

A) Population change after treatment (log CFU/ml)

B) 

|    | Pph | HPL | IR1 | IR2 | IR3 | IR4 | IR5 | IR6 | R1  | R2  | R3  | R4  |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 0  |     |     |     |     |     |     |     |     |     |     |     |     |
| 1/4|     |     |     |     |     |     |     |     |     |     |     |     |
| 1/16|    |     |     |     |     |     |     |     |     |     |     |     |
| 1/64|   |     |     |     |     |     |     |     |     |     |     |     |
| 1/256|  |     |     |     |     |     |     |     |     |     |     |     |
| 1/512|  |     |     |     |     |     |     |     |     |     |     |     |
