1 **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

63 evidence suggests that non-heritable physiological persistence also plays a critical role in both
64 antibiotic treatment failures and infection relapse (3-6). Resistance is a result of genetic changes
65 that enable cells to withstand and grow in the presence of higher doses of an antibacterial agent,
66 whereas persistence is an altered physiological response of a sub-population of genetically
67 sensitive cells to survive transient lethal treatments (7). In addition to the altered physiological
68 response that is mainly governed by gene expression changes, persistence can be caused by
69 genetic mutations in genes associated to these physiological responses. Although resistance and
70 persistence are considered distinct survival strategies, recent evidence suggests that physiological
71 persistence can also reduce antibiotic efficacy by promoting the acquisition of resistance (8, 9).
72 As such, finding alternatives to treat bacterial infections is critical to combat the reduced efficacy
73 of antibiotic therapies. More importantly, employing the alternative treatments in a manner that
74 sustains their efficacy is critical for durable pathogen control. Bacteriocins, which are bacterially
75 produced proteinaceous toxins, have been proposed as antibiotic alternatives (10). However, we
76 currently lack a fundamental understanding of how bacterial populations are likely to survive
77 bacteriocin treatments, should they be widely adopted.

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

82 Bacteriocins are classified into different groups based on their structure, composition, and mode
83 of action. Tailocins are bacteriocins that resemble bacteriophage tails and are grouped into R-
84 type (with a retractile core tube) and F-type (flexible) (14, 15). Bacteria including the
85 opportunistic human pathogen *P. aeruginosa* and other environmental Pseudomonads and

86 *Burkholderia* are known to produce tailocins that are believed to antagonize competitors
87 including pathogenic strains among others (16-22). In fact, a recent study by Principe et al.
88 showed the effectiveness of foliar sprays of tailocins produced by *P. fluorescens* in reducing the
89 severity and incidence of bacterial-spot disease in tomato caused by *Xanthomonas vesicatoria*
90 (22). Other studies have also indicated the potential use of engineered R-tailocins in suppressing
91 food borne pathogens using *in vivo* models (23, 24). Our group has previously characterized a R-
92 type tailocin from a plant pathogenic bacterium *P. syringae* pv. *syringae* (*Psy*) B728a (25). This
93 R-type tailocin showed antagonistic potential against several pathovars of *P. syringae* that cause
94 serious diseases and substantial losses in economically important crops such as common bean
95 (pv. *phasiolicola*), soybean (pv. *glycinae*), chestnut (pv. *aesculi*), and kiwifruit (pv. *actinidae*)
96 (25). A broad spectrum of tailocin mediated antagonistic interaction has been recently described
97 in *P. syringae* (26).

98 In these systems, tailocins are considered to be potent killers as a single tailocin particle is
99 predicted to kill a sensitive cell and an induced cell can release as many as 200 particles (27, 28).
100 R-tailocins kill their targets by binding to the surface receptors and puncturing through the cell
101 membrane causing dissipation of membrane potential and cell death (11, 27). Tailocins are
102 known to recognize and bind specific lipopolysaccharides (LPS) components of the target cells
103 to initiate killing (29-31). LPS is generally composed of three components: the lipid A, core
104 oligosaccharide, and the O-polysaccharide (O-antigen) region (32, 33). The lipid A, and core
105 region are mostly conserved within a species. However, the O-antigen region varies extensively
106 in its chain length and composition of sugars (34). Biosynthesis and transport of LPS to the outer
107 membrane as well the modification of O-antigen involves complex processes involving a number
108 of highly diverse genes (33).

109 Although studies have demonstrated potent bactericidal activity of R-tailocins against pathogenic
110 strains and have suggested their use in pathogen management (25, 26, 35), limited knowledge
111 exists with regard to the efficacy of tailocins to provide sustainable pathogen management.
112 Major limitations related to employing bacteriocins stem from our lack of understanding of how
113 target pathogens might persist in the face of sustained treatment. Moreover, a detailed
114 understanding of the genetics of LPS modification in relation to the evolution of tailocin
115 resistance and persistence in the target pathogen is lacking. This knowledge is fundamental to
116 assessing the sustainability of tailocins as therapeutic agents.

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

131

132 **Materials and Method**

133

134 **Bacterial strains, media, and culture conditions**

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

142 **Tailocin induction and purification**

143 Tailocin or control supernatants for treatment were prepared from logarithmic (log) cultures of
144 *Psy* B728a and ΔRrbp, respectively using a polyethylene glycol (PEG) precipitation protocol as
145 previously described (25, 36). Briefly, 100-fold diluted overnight B728a cultures were sub-
146 cultured for 4-5 hours in KB before inducing with 0.5 μg ml^{-1} final concentration of mitomycin C
147 (GoldBio). Induced cultures were incubated for 24 hours with shaking at 28°C. Next, cells were
148 pelleted by centrifugation and the supernatants were mixed with 10% (w/v) PEG 8000
149 (FisherScientific) and 1M NaCl. Supernatants were then incubated either in ice for 1 hour and
150 centrifuged at 16,000 g for 30 min at 4°C or incubated overnight at 4°C and centrifuged at 7000g
151 for one hour at 4°C. Pellets were resuspended (1/10- 1/20th of the original volume of the
152 supernatant) in a buffer (10 mM Tris PH 7.0, and 10 mM MgSO4). Two extractions with equal

153 volume of chloroform were performed to remove residual PEG. Tailocin activity was confirmed
154 by spotting dilutions of 3-5 µl of both the tailocin and control supernatants onto soft agar
155 overlays of *Pph*. The relative activity of tailocin was expressed as arbitrary units (AU) as
156 obtained from the reciprocal of the highest dilution that exhibited visible tailocin killing in an
157 overlay seeded with ~10^8 CFUs of *Pph* log cells. Lethal killing units of the purified tailocin were
158 determined using a Poisson distribution of the number of surviving colonies after treatment with
159 different dilutions of the tailocin as described previously (21, 37).

160 The relative activity of the purified tailocin was determined to be 10^3-10^4 AU and 1.25×10^7-
161 4.25×10^9 lethal killing units/ml. The minimum inhibitory concentration was estimated to be 100
162 AU when exposed to ~10^6 viable target cells at their logarithmic growth. No loss of tailocin
163 activity was observed for a period of over six months in the buffer (10 mM Tris PH 7.0, and 10
164 mM MgSO4) at 4°C.

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

166 To assess tailocin activity against the stationary and log phase of *Pph*, individual colonies
167 growing on KB agar plates for ~2 days were inoculated into 2 ml of liquid KB medium.
168 Following incubation at 28°C with shaking at 200 rpm overnight, the cultures were back diluted
169 1000-fold into fresh KB. The back diluted cultures were either incubated for 28-30 hours to
170 prepare stationary cultures, or back-diluted 100-fold at 24 hours and cultured for another 4-6
171 hours to prepare log cultures (**see Fig S1** for a growth curve of *Pph*).

172 Stationary cultures were diluted 20,000-fold and logarithmic cultures were diluted 1,000-fold
173 [~10^5 -10^6 CFUs/ml for both cultures **see Fig 1**] in fresh KB before tailocin treatment. Treatment
174 was applied by mixing 10 µl of diluted cultures in 90 µl of purified tailocin preparation diluted in

175 KB. After treatment, samples were incubated for ~ 1 hour at 28°C and washed twice to remove
176 residual tailocin particles. Washing was performed by mixing the treated culture in 900 µl of
177 fresh KB followed by centrifugation at 12,000 g for 2 min. The top 900 µl fraction was discarded
178 and the bottom 100 µl fraction was serially diluted and either spread- or spot-plated to enumerate
179 surviving population. Plates were incubated at 28°C for 2-3 days before enumeration. Serial
180 dilutions of both stationary and log cultures were spotted onto KB agar to enumerate the
181 untreated population. Experiments were performed with various tailocin concentrations (i.e. 100
182 AU, 500 AU, and 900 AU).

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

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

194 **Time dependent death curve with tailocin treatment**

195 Prolonged tailocin exposure was performed with both the stationary and log-phase cultures of
196 *Pph* to generate death curves. Treatment was applied in a 96-well plate as before. Surviving

197 populations were enumerated at 1, 4, 8, and 24 hours following treatment and randomly selected
198 surviving colonies were re-exposed to tailocin to differentiate them into persistent or resistant
199 phenotypes.

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

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

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

206 Stationary and log phase cultures were prepared as described above by culturing *Pph* cells in KB
207 broth for either 28-30 hours or for 4-6 hours, respectively. Cultures were centrifuged for 2 min at
208 12,000 g and the supernatant was filter sterilized using a 0.2 µm syringe filter. Stationary and log
209 supernatants were diluted 1,000-20,000-fold in KB (according to how the cultures were diluted
210 for tailocin treatment). Various dilutions (10-, 50-, 100-, and 1000-folds) of purified tailocin
211 were prepared in the stationary and log supernatants. Dilutions were spotted on a *Pph* lawn using
212 the overlay method. AU of the of the tailocin suspensions were calculated as described above.

213 **LPS extraction and visualization**

214 LPS extraction was performed as described by Davis and Goldberg (38) from pellets of one
215 milliliter of overnight cultures (OD_{600} 0.5). After extraction, 10 µl samples were separated by
216 SDS-PAGE. Samples were labeled using the Pro-Q Emerald 300 glycoprotein stain kit
217 (ThermoFisher Scientific, #P21857) according to manufacturer's instructions. For LPS size

218 determination, CandyCane™ glycoprotein molecular weight standards (ThermoFisher Scientific,
219 #C21852) were included. Gels were visualized using Molecular Imager Gel-Doc XR+ (Bio-Rad)
220 with Image Lab Software.

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

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

231 Resulting forward and reverse read files were paired and mapped to the *Pph* 1448A
232 chromosomal and plasmid sequences using Geneious R10.2 with default parameters for medium
233 sensitivity. Next, genetic variants were identified using 'Find variations/SNPs' program within
234 Geneious using default settings. Regions supported by a minimum of 10 reads and >90% variant
235 frequency were selected. Moreover, variants shared among all the mutants that were generated in
236 independent experiments were discarded as misalignments. Variants identified by Geneious were
237 confirmed in the contigs generated by De Novo assembly of the paired reads using SPAdes
238 3.11.0 using K-mer sizes of 21, 33, 55, 77, 99, 127 with careful mode selected to minimize
239 mismatches and short indels. Indels were also detected and visualized in the contigs by Harvest

240 suit tools (39). Sequencing generated 1,986,400 -2,857,324 paired reads per genome with 48-
241 69X genome coverage. *De novo* assembly generated 308-338 contigs per genome with the N50s
242 of 75,222-81,220bp. The total assembly size was 5.97 Mbp with a GC content of 57.96%, values
243 similar to *Pph* reference genome (40).

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

248 **Statistical analysis**

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

253 **Results**

254 **Tailocin persistence increased in the stationary state**

255 Purified tailocin supernatant was used to test its killing effects on stationary and log phase
256 cultures of the *Pph* target cells in a broth environment. After an hour of 100 AU tailocin
257 treatment, a consistent reduction (3.59 ± 0.12 log reduction) in the viable population occurred for
258 logarithmic cultures, while a significantly lower reduction (1.38 ± 0.14 log reduction) occurred
259 for the stationary cultures. Further analysis showed that, upon treatment of equivalent number of
260 viable cells, stationary cells consistently survived 10 to 100-fold more than the logarithmic cells

261 (**Fig 1** and **Fig S2**). Surviving colonies, especially those from the stationary phase, were
262 predominantly sensitive upon tailocin re-exposure suggesting survival by persistence mechanism
263 (see below).

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

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

275 Upon tailocin re-treatment, >90% of stationary and >60% of log cells that survived the first hour
276 treatment, were as sensitive as the WT (i.e. persistent) as in (**Fig 2B**). The proportion of
277 persistent survivors was higher in the stationary cultures compared to the log cultures at all time
278 points (**Fig 2B**). Tailocin persistent cells were recovered from both cultures even after 24 hours
279 of tailocin treatment, although the proportion decreased over time (**Fig 2A**). Tailocin activity
280 (see **Fig S4**) was detected in the supernatants recovered from the treated samples that contained
281 tolerant cells, confirming saturation of tailocin in the treatment. Although a slight reduction of
282 activity was observed when the tailocin preparation was mixed with undiluted stationary

283 supertant compared to log supertanant, no difference was detected upon diluting the supertants
284 up to 1,000-20,000-fold (as the cultures were diluted for tailocin treatment) before mixing with
285 tailocin (**Fig S5**). This suggested that the increased tailocin tolerance in the stationary phase is
286 not related to inhibition of tailocin activity by an extracellular component.

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

291 **Tailocin exposure selected for heritable mutants showing heterogenous resistance and**
292 **increased persistence**

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

303 LPS analysis of the mutants and the wild-type *Pph* showed that the complete resistant mutants
304 lacked fully-formed O-antigens, whereas the high-persistent and majority of incomplete resistant

305 mutants still possessed the O-antigen with subtle changes. One of the incomplete resistant
306 mutants (IR4), however, showed a very different and faint O-antigen band (**Fig S6**).

307

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

311 Genome sequencing and variant identification of the high-tolerant like (n=1), incomplete
312 resistant (n=6), and resistant mutants (n=4) was performed by mapping the Illumina reads with
313 the parental *Pph* 1448A reference sequence. Mutations were detected in several potential genes
314 responsible for lipopolysaccharide O-antigen biosynthesis in the complete and incomplete
315 resistant mutants (**Table 2**). Mutants isolated at different experiments showed mutation in a
316 different gene. A specific region (**Fig 6**) was identified in the *Pph* genome that showed the most
317 prominent role in tailocin tolerance and resistance. Even within a single gene (PSPPH_0957),
318 different phenotypes were identified depending on the nature of the mutation (**see Table 2 and**
319 **Fig 6).** Moreover, one of the incomplete resistant mutant class (IR6) showed mobilization of the
320 100 bp MITE sequence present in the *Pph* genome as described by Bardaji et al (42). The
321 insertion of this MITE inactivated the target gene (PSPPH_0963) causing the incomplete
322 resistance phenotype. The high tolerant mutant had a 16 bp deletion that caused frameshift in the
323 ORF of a transmembrane domain containing hypothetical protein.

324 **Bioinformatics of mutated genes**

325 Genes identified to have a role in tailocin activity (see **Table 2** for a list) were assessed for their
326 presence and similarity to those found in other *Pseudomonas* genomes. PSPPH_0957 is
327 predicted to encode a glycosyl transferase (family 1) and conferred both complete and
328 incomplete resistance phenotypes depending on the type of mutation (**see Fig 6)** and is present
329 across majority of sequenced *P. syringae* isolates. However, the region from PSPPH_0958 to
330 PSPPH_0964 displays much more variation within *P. syringae.* For instance, a pv. *glycinea* race
331 4 isolate, which is closely related to *Pph,* lacks this region. Although predicted orthologs for
332 some of the genes in this region were present, PSPPH_0963, and PSPPH_0964 that occur
333 contigiously in an operon were less abundant even within *P. syringae* (present only in 87
334 genomes out of 287 *P. syringae* genomes in IMG-JGI database). Orthologs of these two genes
335 were not found in the genomes of pv. tomato (DC3000), *Psy* (B728a)*,* and *Pgy* (R4)*,* but were
336 found in strains of pv. *actinidiae*, pv. *morsprunorum*, pv. *aesculi* and some environmental
337 isolates**.** Although the incomplete resistance gene (PSPPH_0963) was identified to have a
338 FAD/NAD(P) binding cytoplasmic domain with a potential role in LPS biogenesis (IMG
339 product name UDP-galactopyranose mutase), no functional role could be predicted for the
340 hypothetical gene involved in the high-persistent like phenotype. Nevertheless, it possesses ten
341 predicted transmembrane domains and a signal peptide domain at its N-terminus.

342 **Discussion**

343 There has been a renewed research interests in alternative treatment strategies for bacterial
344 pathogens due mainly to the growing threats of antibiotic resistant infections. Tailocins have
345 long been proposed as effective and more specific alternatives to broad spectrum antibiotics.
346 However, the evolutionary dynamics of tailocin persistence and resistance have not been widely
347 investigated. In this study, we addressed this fundamental concern using a phage-tail like

348 bacteriocin (i.e. tailocin) produced by *P. syringae* pv. *syringae* strain B728a in killing target cells
349 of *P. syringae* pv. *phaseolicola* 1448A. Upon expoure of a lethal dose of tailocin to equal
350 numbers of stationary and logarithmic *Pph* cells, a higher fraction of stationary cells survived
351 than cells in log phase. Upon re-exposing surviving cells, a majority of the cells were sensitive to
352 the wild-type level, suggesting that non-heritable persistence rather than heritable resistance was
353 the predominant survival mechanism , particularly in the stationary phase. A prolonged tailocin
354 exposure generated a killing pattern similar to the one reported for persistent sub-population
355 upon antibiotic treatment (7, 43). Persistence was maintained for at least 24 hours with tailocin
356 exposure, a phenomenon that was more evident in some experimental replicates in which
357 resistance evolution was not observed. However, by increasing the tailocin concentration in the
358 treatment, we showed that most of the persistent survivors were killed, and the difference in
359 survival between the two growth phases was no longer seen. Upon re-exposure, however,
360 stationary phase-derived cells exhibited a higher fraction of persistence. Overall, these
361 experiments using various tailocin concentration and exposure time suggested that tailocin
362 persistence is dependent on concentration rather than duration of exposure. We showed that
363 increasing the concentration of the tailocin treatment enabled killing of even the highly persistent
364 stationary cells. This indicated that the stationary cells may require multiple hits by tailocin
365 particles as opposed to the one-hit-one-kill mechanism of killing described for tailocins (27, 28),
366 or that the probabilty of a successful hit in stationary phase is lower than in log phase. Since
367 tailocins are specific to their targets, and are not known to have off-target effects, higher
368 concentration of tailocin could be used to achieve a more effective pathogen control. However,
369 although at a low level, persistence was still maintained even with high-dose tailocin treatment
370 and inherent emergence of either complete or incomplete resistance was frequently observed. As

371 such, although a significant reduction in pathogen population and disease pressure can be
372 obtained with tailocins, a stand-alone tailocin treatment might not be enough to achieve a
373 sustainable pathogen control.

374 The use of the term 'persistence' in relation to antimicrobial survival is disputed to some extent
375 and is sometimes used interchangably with 'tolerence' and 'viable but not culturable state'. In
376 this paper, we used the term 'persistence' as this trait was only seen in a sub-population, resulted
377 in a bi-phasic death curve, the surviving persister cells resuscitated in the absence of the tailocin
378 treatment, and were equally sensitive to the wild-type cells upon re-exposure. This definition of
379 persistence has been suggested previously (7). Persistence to antimicrobials is being increasingly
380 recognized for its role in antimicrobial treatment failures with bacterial infections (44). Various
381 mechanisms such as formation of persister-subpopulation or biofilms are implicated in the
382 maintainance of persistence (45, 46). However, persistence responses can be different based on
383 the stresses involved and their mode of action as reported previously with different antibiotic
384 treatments (47). Several toxin-antitoxin (TA) systems are implicated in the formation of
385 persister-subpopulation (4, 6, 48). TA systems were shown to be induced when cells were
386 starved for certain sugars and amino acids or by exposure to osmotic stresses that altered ATP
387 levels in the cell (47, 49). However, TA system activation did not always induce persister
388 formation (49). Additionally, recent findings have indicated a mechanism mediated by the
389 guanosine pentaphosphate/tetraphosphate (ppGpp) for persister formation that is not dependent
390 on a TA system (50). Instead, a strong stationary state effect, that likely involved starvation
391 response, was shown to increase persistence by 100-1,000 fold in *Staphylococcus aureus* with
392 ciprofloxacin treatment (49). Whether similar mechanisms of TA and/or ppGpp systems regulate
393 tailocin persistence or a specific mechanism for tailocin and/or related bacteriophage exists,

394 remains to be determined. Nevertheless, our data of the difference in tailocin tolerance between
395 the stationary and log cultures suggests that metabolic inactivity and starvation induced stress
396 could be a strong factor in tailocin persistence.

397 Few previous studies have demonstrated growth-phase dependent differences in LPS O-antigen
398 chain length and composition or their regulatory pathways (51, 52). Furthermore, in a previous
399 study with *P. fluorescens,* exponentially growing cells had a significantly higher rate of cell lysis
400 than stationary or decline phase cells with bacteriophage PhiS1 (53). It could be possible that the
401 stationary phase cells possess an altered O-antigen that interferes with tailocin binding and target
402 recognition, causing the difference in tailocin survival between the two growth phases. This
403 could be supported by our observation that un-diluted stationary phase supernatant inhibited
404 tailocin activity to some extent compared to the log phase supernatant. Moreover, the finding that
405 mutation in one of the hypothetical proteins containing a signal peptide and several trans-
406 membrane domains caused increased tailocin persistence is noteworthy. More importantly, the
407 stationary and log state difference in tailocin survival was lost for this mutant. Since the
408 hypothetical protein occurs in the same operon as other LPS biogenesis genes, it is likely that it
409 plays a role in O-antigen biogenesis and maintenance, thereby reducing tailocin interaction with
410 the cells.
411 Another mechanism that can cause increased survival to surface active antimicrobials (eg.
412 phages and host immune defenses) is phase variation (54). Phase variation is a gene regulation
413 system that induces heterogenous expression of specific genes in a clonal population (54-56).
414 Phase variation is heritable but reversible as the cells go through generations. The 'ON' 'OFF'
415 switch occurs randomly amounting to 10^{-4} to 10^{-1} per generation, significantly greater than is
416 expected by mutational events (57). Phase variation has been shown to modify LPS operons in *S.*

417 *enterica* spp (58). Moreover, temporal development of phage resistance in *S. enterica* serovar
418 Typhimurium by phase variable glucosylation of the O-antigen has been evidenced (59). In
419 addition, phase variation in *P. fluorescence* has been demonstrated during rhizoshpere
420 colonization of Alfala. The phase variants were enhanced in motility by production of a longer
421 flagella than the wild type cells and were able to colonoze distal end of the roots (60). It can be
422 expected that phase variation could be a part of tailocin survival mechanism, particularly because
423 of the fact that LPS, that is commonly modified in bateria by phase variation, serves as the
424 receptor for tailocins. Previous studies with treatments of bacteriophage that recognize the target
425 cell LPS have evidenced the role of phase variation in phage defense (61, 62), including in the
426 closely related *P. aeruginosa* (63). However, whether tailocin persistence observed here is the
427 result of phase variable LPS genes, needs further assessement. Nevertheless, in one of the
428 incomplete resistant mutants, the target gene was modified by movement and integration of an
429 internal mobile genetic element (MGE). MGEs have been described to have a role in causing
430 phase variation in other bacterial systems (64, 65). Further highthroughput screening techniques
431 that can test the sensitivities of thousands of progeny CFUS of the incomplete resistant mutants
432 will be required to determine the rate of switch to confirm if this is a case of phase variation.

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

440 level, unlike in the complete resistant mutants that had completely lost their O-antigen.
441 Preserving the O-antigen and still surviving the tailocin or phage attack may prevent the fitness
442 cost associated with complete resistance evolution. Further tests that will compare the virulence
443 and fitness traits of the incomplete resistant mutants together with the wild-type and resistant
444 lines will be required to confirm these hypotheses.

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

454 Although LPS O-antigens are known to be tailocin receptors, the involvement of specific genes
455 and LPS regions in tailocin recognition has not been clarified in *P. syringae*. In this study, we
456 identified several genes of LPS O-antigen biosynthesis, and showed that mutations of some of
457 these genes causes complete loss of O-antigen and therefore complete tailocin resistant mutants.
458 On the other hand, other mutations at the same or different genes cause potential change in the
459 O-antigen chain lengh and composition causing incomplete resistance. Some complete resistant
460 mutants showed a typical rough-colony morphotype due to lack of O-antigen. Rough-colony
461 morphotypes lacking O-antigen were resistant to phages as described previously in *P. syringae*
462 pv. *morsprunorum* isolates from plum and cherry (73). Moreover, these rough colony

463 morphotypes showed reduced virulence in the plants (73). In other plant pathogenic bacteria such
464 *as Xanthomonas* oryzae pv. oryzae and *Xylella fastidiosa,* loss of O-antigen, respectively,
465 reduced type III secretion into plants (74), and increased recognition of the pathogen by the host
466 immune response (75). In both cases, plant virulence of the mutants was significantly reduced
467 (74, 76). This sugests that the complete tailocin resistant mutants that lack the fully-formed O-
468 antigen might, in fact, loose plant fitness. Other means of survival such as tolerance, and
469 incomplete resistance described here might therefore serve to counter the fittness cost associated
470 with gaining complete tailocin resistance, enabling survival from the attack of both the co-
471 infecting competitiors as well as host defense responses.

472

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686 **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)

687

688 **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	-	-	
HPL	2.95±0.36	PSPPH_0964	Hypothetical protein	16 bp deletion, frameshift	Low
IR1 (BRM279)	0.48±0.08	PSPPH_3226	Glycosyl transferase	T>P	High
IR2 (BRM233)	0.78±0.28	PSPPH_2810	4-amino-4-deoxy-L-arabinose transferase	S>I	High
IR3 (I3)	0.33±0.14	PSPPH_0960	Glycosyl transferase family 2	(N>I)	High
IR4 (BRM11)	-0.10±0.05	PSPPH_0957	Glycosyl transferase	T insertion, extra AA added at the end	High
IR5 (BRM334)	-0.1±0.07	PSPPH_0520	Glycosyl transferase	H>R	High
IR6 (I6)	-0.260±0.06	PSPPH_0963	FAD- dependent oxidoreductase	100 bp insertion,	Low

				truncated protein (mite)	
R1 (BRM16)	-0.26±0.06	PSPPH_0957	Glycosyl transferase	C>A substitution, truncated protein	High
R2 (BRM15)	-0.17±0.08	PSPPH_0957	Glycosyl transferase	E>K	High
R3 (BRM258)	-0.19±0.15	PSPPH_0952	Nucleoside-diphosphate-sugar epimerase	R>W	High
R4 (BRM328)	-0.22±0.17	PSPPH_0983	Glycosyl transferase	Frameshift	High

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690

691 **Figure legends**

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

697 **Fig 2. Time dependent dynamics of tailocin survial**. **A)** Stationary and log cultures of *Pph*
698 were treated with tailocin and the viable population was enumerated before and 1, 4, 8, and 24
699 hours post-treatment. Three independent experiments were performed with 3-6 biological
700 replicates per time. Mean and standard error of mean are graphed. Different letters represent
701 significant differences as analyzed in SAS 9.4 with proc Glimmix at $P=0.05$. **B)** Percentage of
702 surviving colony phenotye upon tailocin re-treatment. Randomly selected surviving colonies

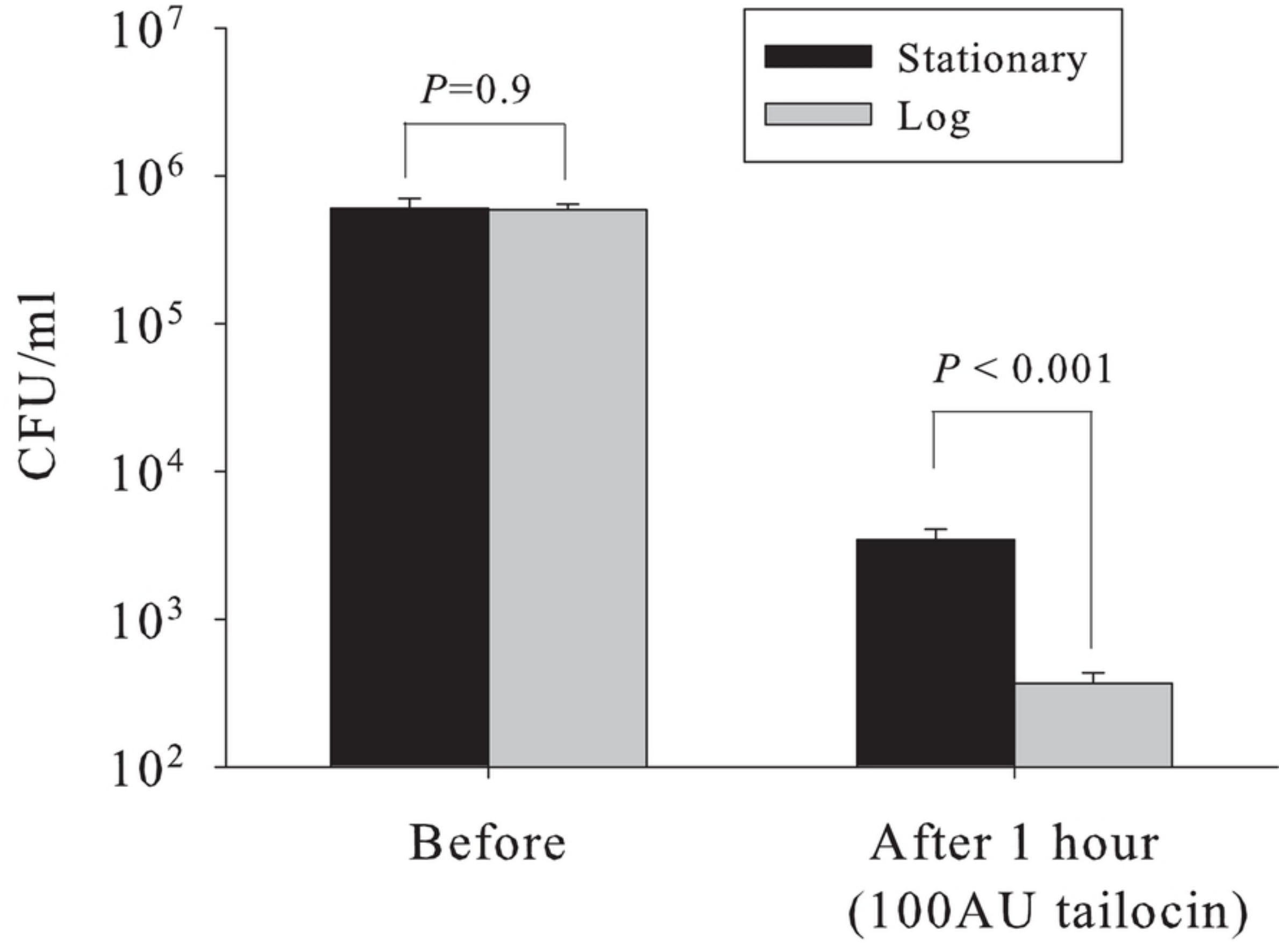
703 (n=12-44 for each growth phase and hours of treatment) from the initial tailocin treatment were
704 sub-cultured and re-treated with tailocin, and percentage of the surviving phenotype was
705 calculated. Colonies recovered from three independent experiments were used for the re-
706 treatment to calculate this percentage.

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

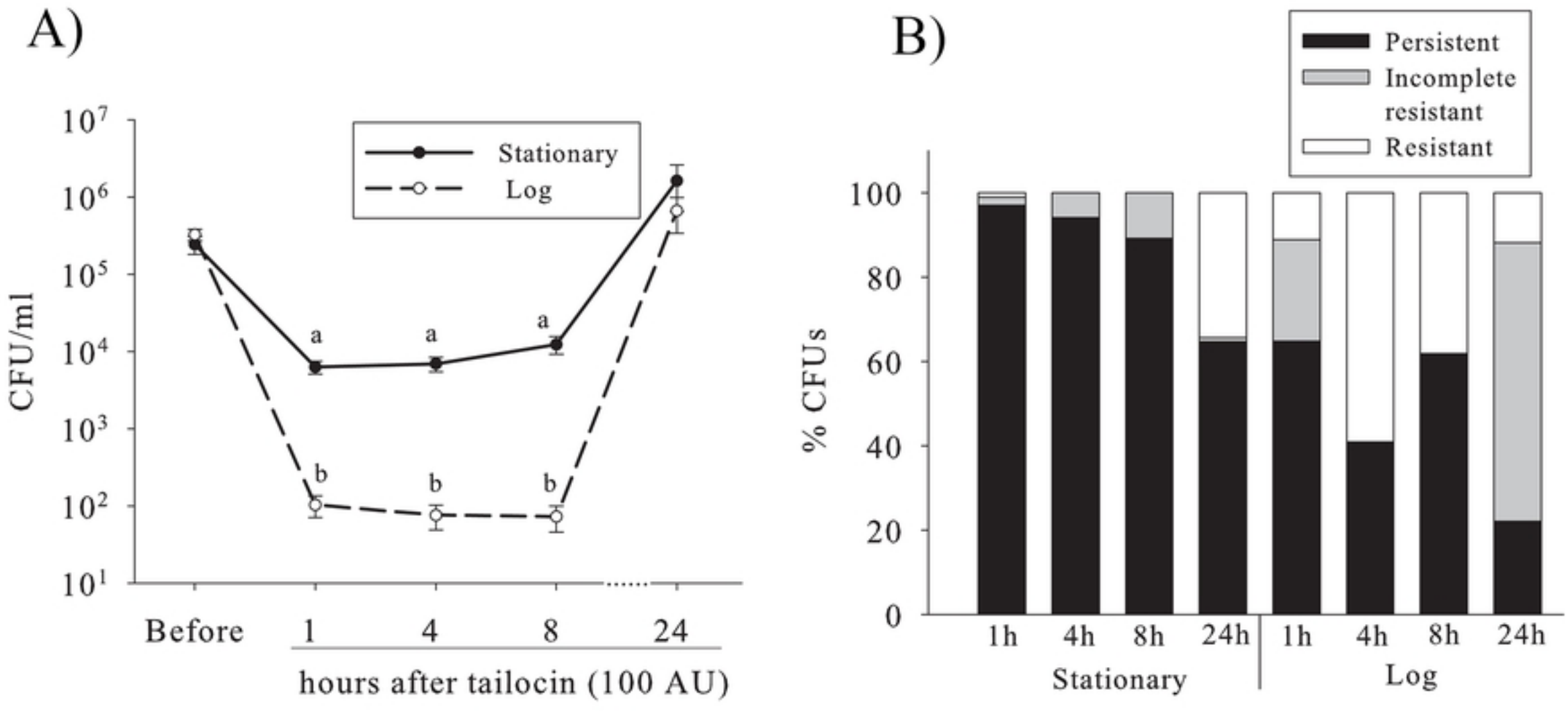
716 **Fig 4**. **Treatment response of tailocin persistent and resistant lines. A)** Reduction in the
717 population of tailocin persistent and resistant mutant lines upon re-treatment with tailocin. Log
718 cultures of each lines were treated with 900 AU of tailocin and change in the population was
719 calculated after an hour of tailocin treatment. At least three separate colonies of each lines were
720 tested and experiments were repeated a minimum of three times. Means of the difference in log
721 transformed viable population pre- and post treatment are graphed. Error bars indicate stantard
722 error of mean. **B)** Assessement of response of mutant lines to tailocin under overlay condition.
723 Dilutions of tailocins (shown on the left most column) were spotted over the culture lawn of each
724 of the lines. Yellow line indicates the dilution upto wchich visible killing was observed. HPL;
725 high persistent-like, IR; incomplet resistant, R; resistant.

726 **Fig 5. Dynamics of tailocin survival for the high persistent-like (HPL) mutant.** Cultures
727 were treated with various tailocin doses and viable cells pre- and post-treatment were
728 enumerated. Means and standard error from three independently repeated experiments with at
729 least three biological replicates per experiment are reported. More killing occurred at high
730 tailocin concentration and no difference in stationary and log phage was observed. $P>0.05$
731 indicate no significant differences within grouped bars as analyzed in SAS 9.4 with proc
732 Glimmix.
733
734 **Fig 6**. **Mapping a genomic region (PSPPH_0957- PSPPH_0964) of *Pph* that showed**
735 **prominent effect on tailocin sensitivity and resistance**. Mutations of this region gave rise to
736 multiple phenotypes that differed in tailocin tolerance/resistance. Refer Table 2 for a complete
737 list of genes at this and other genomic locations.

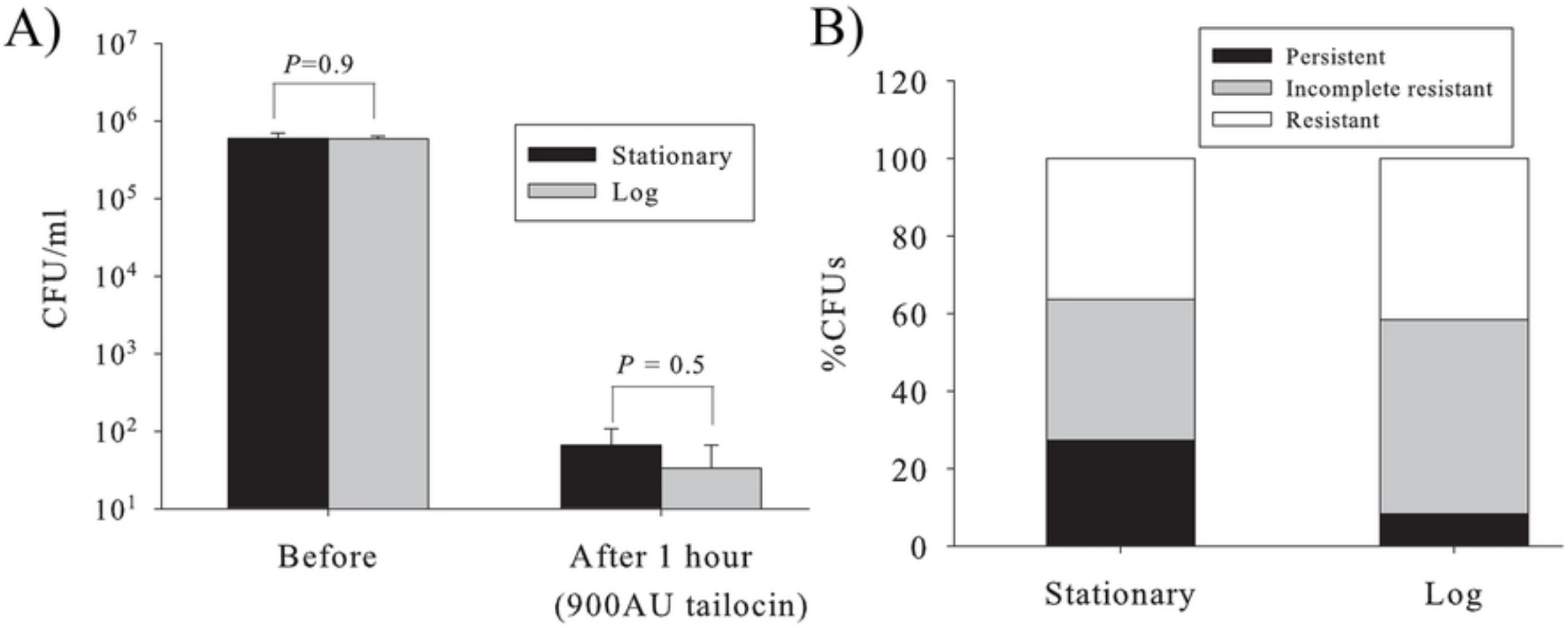
738


Stationary
Log
P=0.9
P < 0.001
CFU/ml
10^7
10^6
10^5
10^4
10^3
10^2
Before
After 1 hour
(100AU tailocin)

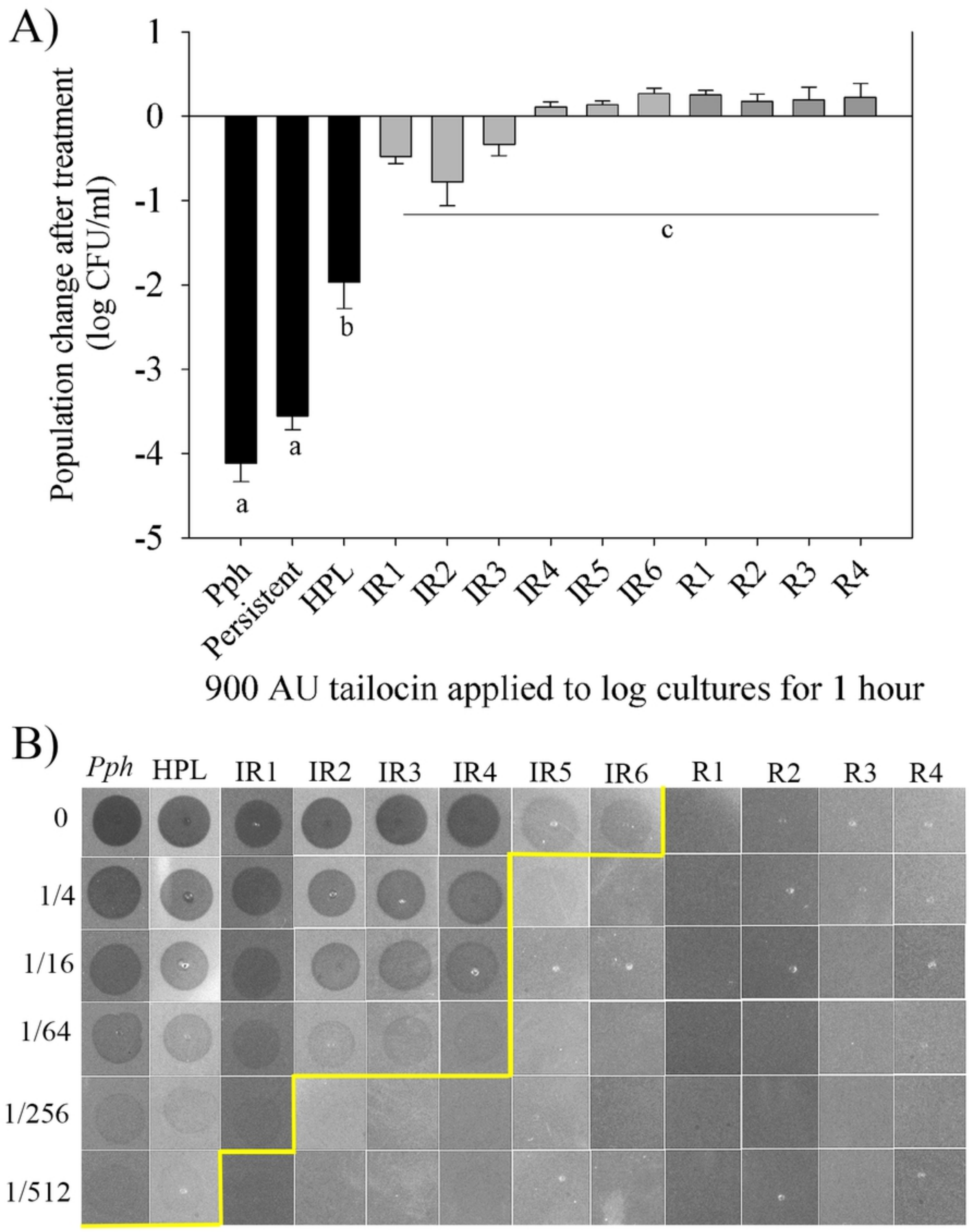
Figure


A)
Stationary
Log
CFU/ml
10^7
10^6
10^5
10^4
10^3
10^2
10^1
a
a
a
b
b
b
Before
1
4
8
24
hours after tailocin (100 AU)
B)
Persistent
Incomplete resistant
Resistant
% CFUs
100
80
60
40
20
0
1h
4h
8h
24h
1h
4h
8h
24h
Stationary
Log

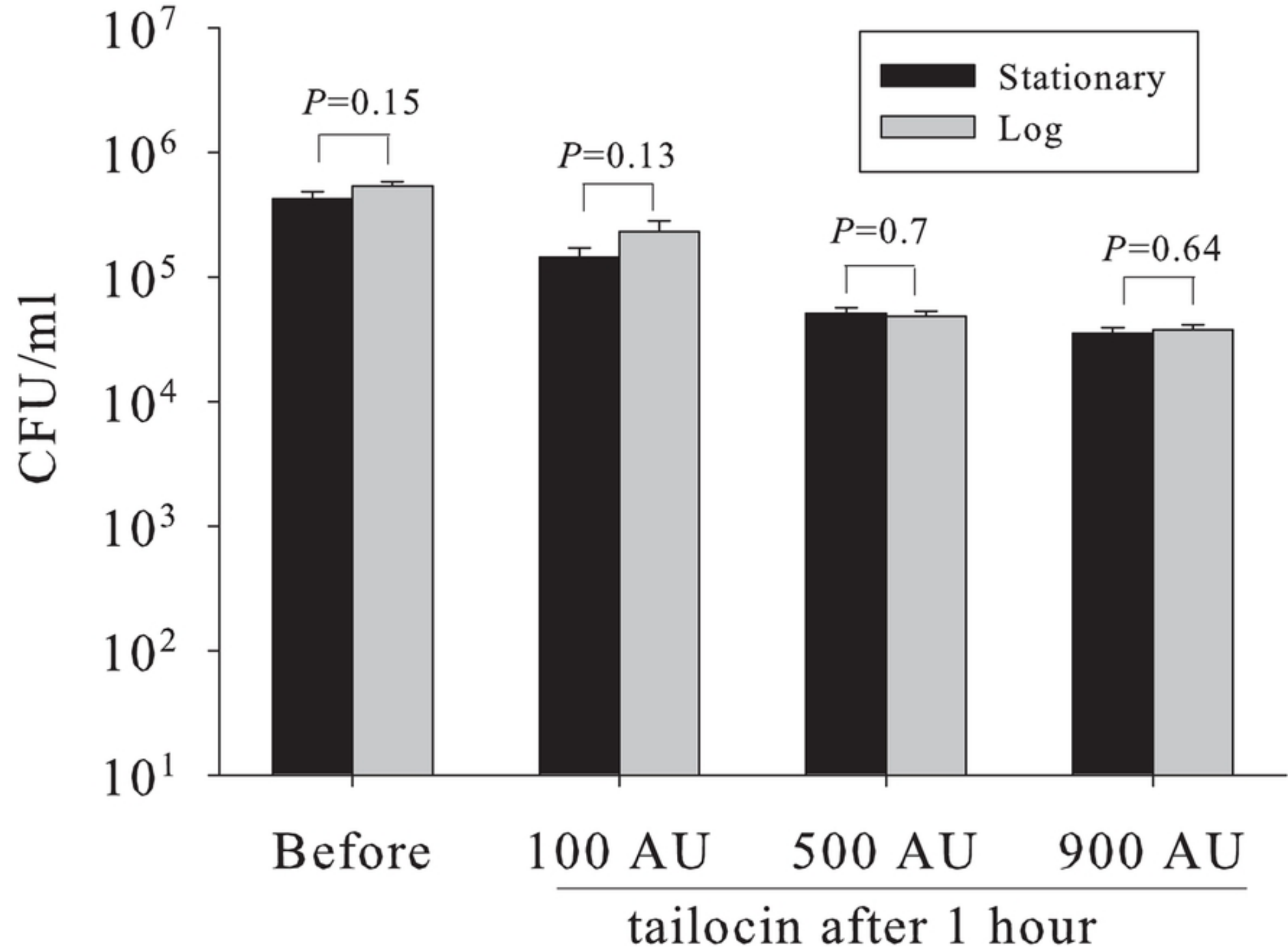
Figure


A)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
CFU/ml
P=0.9
P = 0.5
Stationary
Log
Before
After 1 hour
(900AU tailocin)
B)
120
100
80
60
40
20
0
%CFUs
Persistent
Incomplete resistant
Resistant
Stationary
Log

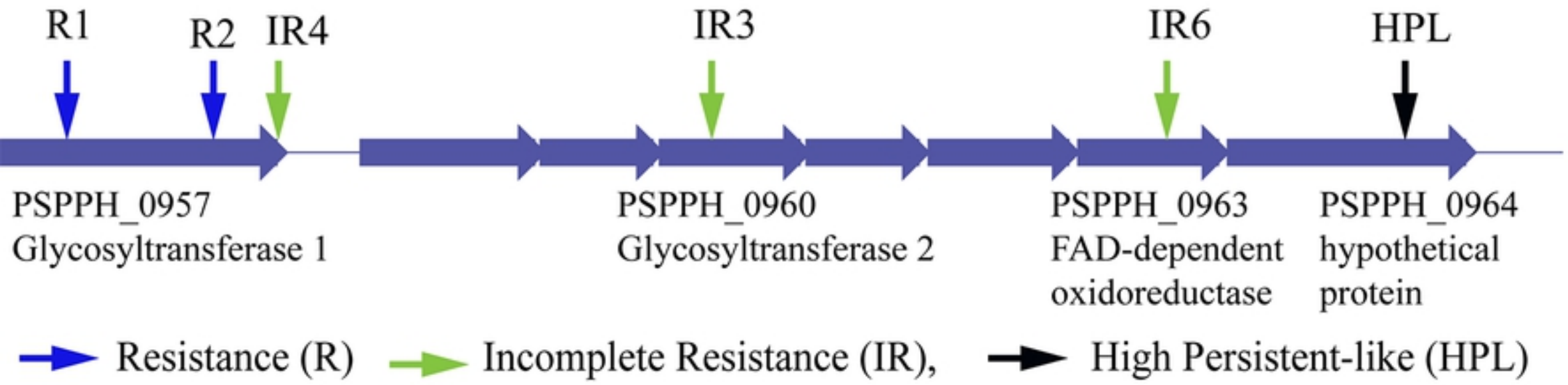
Figure


A)
Population change after treatment (log CFU/ml)
1
0
-1
-2
-3
-4
-5
a
a
b
c
Pph
Persistent
HPL
IR1
IR2
IR3
IR4
IR5
IR6
R1
R2
R3
R4
900 AU tailocin applied to log cultures for 1 hour
B)
Pph
HPL
IR1
IR2
IR3
IR4
IR5
IR6
R1
R2
R3
R4
0
1/4
1/16
1/64
1/256
1/512

Figure


Stationary
Log
P=0.15
P=0.13
P=0.7
P=0.64
CFU/ml
10^7
10^6
10^5
10^4
10^3
10^2
10^1
Before
100 AU
500 AU
900 AU
tailocin after 1 hour

Figure


R1
R2
IR4
IR3
IR6
HPL
PSPPH_0957
Glycosyltransferase 1
PSPPH_0960
Glycosyltransferase 2
PSPPH_0963
FAD-dependent
oxidoreductase
PSPPH_0964
hypothetical
protein
Resistance (R)
Incomplete Resistance (IR),
High Persistent-like (HPL)

Figure