A *Pseudomonas* plant pathogen uses distinct modes of stationary phase persistence to survive bacteriocin and streptomycin treatments

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Abstract

Antimicrobial treatment of bacteria often results in a small population of surviving tolerant cells, or persisters, that may contribute to recurrent infection. Antibiotic persisters are metabolically dormant, but the basis of persistence to membrane-disrupting biological compounds is less well-understood. We previously found that the model plant pathogen *Pseudomonas syringae* pv. *phaseolicola* 1448A (*Pph*) exhibits persistence to tailocin, a membrane-disrupting biocontrol compound with potential for sustainable disease control. Here we compared physiological traits associated with persistence to tailocin and to the antibiotic streptomycin, and established that both treatments leave similar frequencies of persisters. Microscopic profiling of treated populations revealed that while tailocin rapidly permeabilizes most cells, streptomycin treatment results in a heterogeneous population of redox and membrane permeability states. Sorting cells according to redox reporter intensity identified streptomycin persisters among the low-redox fraction, but tailocin persisters were only cultured from the fraction with intermediate redox activity. Cells from culturable fractions were able to infect host plants, while nonculturable redox-active cells were not. Tailocin and streptomycin were effective in eliminating all persisters when applied sequentially, in addition to eliminating cells in other viable states. This study identifies distinct redox states associated with antibiotic persistence, tailocin persistence, and virulence, and demonstrates that tailocin is highly effective in eliminating dormant cells.

Importance

Populations of genetically identical bacteria encompass heterogeneous physiological states. The small fraction of bacteria that are dormant can help the population survive exposure to antibiotics and other stresses, potentially contributing to recurring infection cycles in animal or plant hosts. Membrane-disrupting biological control treatments are effective in killing dormant bacteria, but these treatments also leave persister-like survivors. The current work demonstrates that in *Pph*, persisters surviving treatment with membrane-disrupting tailocin proteins have an elevated redox state compared to dormant streptomycin persisters. Combination treatment was effective in killing both persister types. Culturable persisters corresponded closely with infectious cells in each treated population, whereas high-redox and unculturable fractions were not infectious. In linking redox states to heterogeneous phenotypes of tailocin persistence, streptomycin persistence, and infection capability, this work will inform the search for mechanisms and markers for each phenotype.

Introduction

The phenomenon of bacterial physiological tolerance to antibiotics is a long-standing problem in treating infection. The small fraction of cells in a bacterial population that survive after sustained lethal doses of antibiotics, termed persister cells, are a potential source of recurrent infections (1) or new resistance mutations (2, 3). Unlike genetically resistant cells, persisters occupy a low-metabolic state that is both nonheritable and reversible, where both growth and susceptibility are regained following antibiotic removal. The persister state may be induced in response to stress or stochastic variation in gene

expression, and occurs at increased frequency in stationary phase populations due to nutrient starvation (4). Persistence is just one of several states of bacterial dormancy proposed in the literature (5), and researchers have observed phenotypic overlaps between persisters and viable but nonculturable (VBNC) cells, defined as living cells which are not revivable in standard media (6, 7). Single cell observation after live/dead vitality staining has been a useful strategy to phenotype heterogeneous populations, previously demonstrating that both antibiotic persisters and starvation-induced VBNC cells occupy a low-redox state (8), and that VBNC cells outnumber persister cells after antibiotic treatment (9). The low metabolic rate of persisters and VBNC cells confers protection from antibiotics that target active processes (10).

Physiological tolerance may be an important issue in controlling bacterial plant diseases, which cause significant economic losses (11). Plant pathogenic bacteria face a wide variety of stresses including antimicrobial treatments, extremes in temperature, dessication, nutrient starvation, and host redox defenses. VBNC cells are well-documented in plant pathogens; nonculturable populations arise in response to plant or environmental conditions and can revive to initiate novel infections (12-16). The role of culturable persisters in plant disease is not well understood, but at least two phytopathogen species have been observed to form persisters to the aminoglycoside antibiotic streptomycin and to tetracycline (17, 18). Streptomycin and other antibiotics have been used for plant disease prevention since the 1950s, and extension records indicate that 8 antibiotic classes are currently applied to crops in different regions of the world (19, 20). Antibiotic use is now restricted to a few specific applications in the US, but the more than 40 tons of aminoglycosides annually applied to plants still far exceeds the amount used in clinical medicine (20, 21). The prevalence of persistence, and its potential impact on disease recurrence and management, is an important question in phytopathology.

A promising strategy for eradicating antibiotic persisters is combination treatment with compounds that attack static structures rather than growth processes, including membrane-disrupting polymyxin antibiotics such as colistin (10). Candidate biological control treatments including phage hydrolases, bacteriocins, and antimicrobial peptides also disrupt bacterial membranes, and are also effective at killing antibiotic persisters (22-25). Bacterial subpopulations can also survive membrane disrupting treatments in a conditional or nonheritable fashion (25-28). Investigating the frequency and biological basis of phenotypic tolerance to membrane disruptors will be important to understand the limits and extend the durability of these biocontrol strategies. Tailocins, or phage tail-like particles that disrupt the membranes of the target cell, are a class of bacteriocins with promise for highly specific control of plant disease (29). A tailocin purified from *Pseudomonas syringae* pv. *syringae* strain B728A efficiently kills the model bean pathogen *P. syringae* pv. *phaseolicola* strain 1448A (also referred to as *P. savastanoi* pv. *phaseolicola* in the literature, henceforth *Pph*). This and other tailocins prevent disease when applied prophylactically to plants (30-32). We recently found that a small fraction of the *Pph* population escapes lethal doses of tailocin *via* a nongenetic persister-like mechanism, and heritable tailocin resistance repeatedly arises in culture from the surviving population (30). We termed this “tailocin persistence” in light of its biphasic killing pattern.

In this study, we asked whether tailocin persistence in *Pph* is distinct from persistence to antibiotics, and hypothesized that tailocin persisters could be eliminated through combination therapy with antibiotics. We first established that *Pph* exhibits persistence to streptomycin, then used redox and membrane integrity reporter dyes to determine that tailocin treatment is much more efficient than streptomycin in rapidly eliminating viable cells from the unculturable population. Cell sorting analysis determined that culturable tailocin persisters have a higher level of redox activity compared with those surviving streptomycin, and combination treatment with streptomycin and tailocin eliminated both culturable and nonculturable viable cells. Moreover, we found that culturable persisters to each treatment were able to infect host plants, while nonculturable redox-active cells were not. This study demonstrates a distinct physiological state of persistence to an effective membrane disrupting biocontrol agent, and establishes a foundation for future studies toward identifying persister eradication mechanisms.

Results

Streptomycin and tailocin treatments yield similar frequencies of *Pph* persisters

We previously found that tailocin exposure results in a stable population of genetically susceptible *Pph* survivors, which we defined as tailocin persisters (30). In this study, we asked whether *Pph* exhibits persistence to the antibiotic streptomycin, and whether streptomycin persistence is distinct from tailocin persistence. *Pph* growth curves were performed to establish the timing of early and late stationary phase (Fig. S1). Survival to 5X MIC streptomycin and tailocin was characterized through kinetic killing curve assays at log phase, early stationary phase (20h), and late stationary phase (96h, Figure 1). In both early and late stationary phase cultures, CFU counts declined to 0.04% of initial values within 3h of streptomycin treatment, remaining stable at subsequent timepoints (Fig. 1A). Similarly, 0.06% of early or late stationary phase *Pph* cells remained culturable after tailocin exposure, consistent with our previous observations, with the majority of killing occuring within a few minutes (Fig. 1B). Adding either treatment in log phase resulted in a lower proportion of survivors. For each assay, colonies from the surviving population were confirmed to be susceptible upon re-exposure to streptomycin and tailocin. These results demonstrate that stationary phase *Pph* populations form similar proportions of culturable persisters to both streptomycin and tailocin, although tailocin killing is far more rapid. Subsequent experiments in this study were performed on early stationary phase *Pph* unless otherwise noted, using the same doses and treatment durations as used for the experiments in Figure 1.

Streptomycin and tailocin treatments have distinct effects on population physiology

Having established that tailocin and streptomycin both eliminate most culturable cells, we next sought to compare their efficiency in eliminating the viable population, inclusive of nonculturable cells. We first measured treatment-induced changes in total cell concentration and culturable frequency. Hemocytometer readings revealed that streptomycin did not cause a reduction in the total concentration of cells in stationary phase or log phase (Fig 2A, Fig. S2A). Tailocin caused a slight reduction in the number of cells, although this was only statistically significant in log phase (Fig 2A, Fig. S2B). Dilution

plating confirmed that treatments reduced the proportion of culturable cells from 46% to 0.04% for streptomycin (Fig. 2A) and to 0.05% for tailocin (Fig. 2B), consistent with our earlier measures of survival over the T0 population (Fig. 1). To compare the physiological states of streptomycin- and tailocin-exposed *Pph* populations, we imaged cells on an agarose pad after staining with a combination of three fluorescent dyes: the vitality indicator Redox Sensor Green (RSG), the red membrane permeability indicator Propidium Iodide (PI), and the blue membrane permeant nucleic acid stain Hoescht 33342. This strategy allowed imaged cells to be classified into five categories (Fig. 2C): 1) redox-active with intact membranes (green/blue), 2) redox-active with compromised membranes (green/red/blue), 3) redox-inactive with compromised membranes (red/blue), 4) redox-inactive with intact nuclear material and membranes (blue), and 5) unstained “ghost” cells with no nucleic acid content, visible in phase-contrast only. The method was first tested on log-phase and ethanol-killed cells to rule out signal interference or overlap between stains (Fig. S3), and we confirmed that the staining combination did not affect culturable rate of streptomycin-treated cells. In preliminary experiments we noted that all *Pph* cells showed permeabilization and loss of redox activity starting after two hours on the agarose pad, thus all imaging was performed within twenty minutes after placement on the pad.

In untreated stationary phase cultures, over 80% of the population was composed of redox active, or Category 1, cells (Fig. 2D-E, Table S1). After streptomycin treatment, *Pph* cultures were evenly distributed through Categories 2-4, each comprising an average of 19-25% of the population. Notably, nearly half the treated population were redox-active cells with permeable membranes (Category 2) or redox-inactive cells with intact membranes (Category 4), two states not distinguished by common live-dead staining methods. Cells in Category 2 had a lower green signal intensity than those in Category 1, indicating that membrane-damaged active cells were associated with reduced redox activity (Fig. S4A). A Category 4-like state was previously associated with persister and VBNC cells in *E. coli*, which were also characterized by increased cell roundness (7); we determined that Category 4 individuals also had significantly increased average roundness compared with the four other categories of treated *Pph* cells (Fig. S4B).

Unlike streptomycin, tailocin treatment converted most of the stationary phase population to Category 3 (membrane compromised, inactive) within three minutes (Fig. S5). After four hours, only 3% of the remaining cells were in Category 1 (Fig. 2E, Table S1), and Category 4 cells were extremely rare. When the experiments were performed on cultures in log phase, streptomycin and tailocin-induced changes were similar to those in stationary culture, although there was an apparently reduced proportion of redox-active Category 2 cells after tailocin treatment compared to the log phase results (Fig. S2D). Together, the results indicate that streptomycin treatment shifts the majority of the *Pph* population into diverse physiological states, while tailocin treatment rapidly compromises redox activity and membrane integrity in the vast majority of the population. They also show that after either treatment, the proportion of redox-active cells and other intact cells far exceeds that of culturable persisters.

Streptomycin and tailocin culturable persisters occupy distinct physiological states

While microscopic studies were useful for profiling redox and permeability changes following either treatment, this approach could not determine the culturability of each staining category. Therefore, we applied fluorescence-assisted cell sorting (FACS) to determine whether the culturable and infectious fractions of streptomycin and tailocin-treated populations could be separated according to redox staining characteristics. Because propidium iodide had stained some redox-active *Pph* cells (i.e., Category 2 cells), we first sought an alternate permeability stain that could provide a distinct live-dead separation in two-color sorting studies. DRAQ7 is a far-red membrane permeant dye that has been validated in eukaryotic cell culture studies (33), but is not widely used for determining viability in bacteria. In microscopic analysis on *Pph*, DRAQ7 stained redox-inactive cells, but unlike PI, was not observed to co-stain with RSG (Fig. S6). This indicated that DRAQ7 does not permeate cells with redox activity. A triple staining experiment using DRAQ7 instead of PI was performed on treated and untreated *Pph* to confirm that the stain yielded similar estimates of redox-inactive membrane compromised cells to PI (Table S2).

Flow cytometric analysis of untreated log and stationary phase, ethanol-killed, and unstained cells identified clear patterns associated with death and active growth (Fig. 3A-3D). Consistent with microscopic observations, streptomycin treatment resulted in an apparent increase in both permeabilized cells and low-redox intact cells, but also resulted in a large population of cells with elevated redox signal (Fig. 3E). As expected, tailocin treatment permeabilized all but a small fraction of cells to DRAQ7 (Fig. 3F). To determine how the streptomycin-induced changes compared with the effects of a validated persister induction treatment, we also treated stationary cells with the protonophore CCCP, a highly efficient inducer of multidrug-tolerant persisters in *Pseudomonas aeruginosa* (34). A killing curve assay demonstrated that 3h CCCP treatment (5x MIC, or 100 μg mL^{-1}) resulted in a stable culturable population representing 13% of the initial count (Fig. S7). CCCP treated cultures showed slightly increased RSG staining in intact cells, with many cells permeabilized to DRAQ7 (Fig. 3G). Histogram analysis supported the finding that streptomycin and CCCP treatments generated populations with increased redox signal, while tailocin treatment largely abolished redox activity (Fig. 3H). For unknown reasons, ethanol-killed cells had a higher level of green fluorescence in cell sorting than tailocin-permeabilized cells (Fig. 3H).

Cells were separated according to physiological state to determine the culturability of each fraction. Optimization assays confirmed that no culturable cells could be recovered from DRAQ7-staining fractions, so we focused on the region of low DRAQ7 intensity. Cells were gated into fractions G1, G2, and G3, corresponding to the highest to lowest green fluorescence intensity (Fig. 3A and 3D-G). Cells intact after streptomycin, tailocin, and CCCP treatments primarily fell into the G1, G3, and G2 gates, respectively (Fig. 3E-G and Table S1). Cells collected from each sorting gate were plated on culture media. In untreated cultures, colonies were recovered from all fractions (Fig. 4A). After streptomycin treatment, over 99.5% of colonies recovered came from the low-redox G3 fraction (Fig. 4B), even though this fraction represented only 13% of gated cells (Fig. 3). In contrast, in the tailocin-treated culture over 99% of colonies were recovered from the G2 fraction (Fig. 4C), despite this fraction containing only 0.2% of the total gated cells (Fig. 3). Colonies were cultured from both G2 and G3 fractions after CCCP treatment (Fig. 4D), but similarly to the other two treatments, nothing was cultured from the G1 fraction. These results

demonstrate that while streptomycin persisters occupy a low-redox state consistent with dormancy, tailocin persisters are associated with a state of moderate redox activity. Additionally, diverse treatments resulted in a lack of culturability in cells with high redox activity.

We next asked whether the culturability or redox activity of sorted fractions was associated with infectious capacity of the pathogen. Due to the low volume of sorted inoculum, pathogenicity of the fractions was assessed in a qualitative bean pod inoculation assay, and symptoms of watersoaking or necrosis were observed after five days (Fig 4E-H). No symptoms developed after inoculation from the high-redox G1 fraction of any culture, even without antimicrobial treatment (Fig. 4E). For cultures treated with streptomycin, tailocin, or CCCP, symptoms were observed at sites inoculated with any fraction with a significant culturable population (~10^4 or greater CFU mL^{-1}, 4F-H). Symptoms were weakest in the tailocin treated cultures, but this may be attributable to the low number of culturable cells obtained through sorting. Notably, for streptomycin and tailocin-treated cultures, the fractions associated with the largest number of membrane-intact cells (G1 and G3, respectively) did not cause symptoms (Fig. 3E-F and 4F-G). These results demonstrate that culturability in media is associated with infection capacity in antimicrobial stressed *Pph*, and that the highest redox fractions of all cultures were noninfectious.

Streptomycin and tailocin-treated cells colonize the host at the same rates as untreated cells

Because streptomycin persisters were associated with low activity, we hypothesized that streptomycin persisters might colonize the plant at a slower rate than the more active tailocin persisters. Sorting did not yield a sufficient number of cells to perform timepoint analysis, so we instead compared colonization rates of treated and untreated cultures that had been adjusted to contain the same concentration of culturable cells (2.5 ×10^4 CFU mL^{-1}). Because streptomycin causes a vast decline in culturable cells, the streptomycin-treated inoculum contained roughly 700-fold more RSG-staining cells than the untreated inoculum. The tailocin inoculum contained a similar number of RSG-staining cells to the untreated inoculum, but a far greater number of permeabilized cells. Despite differing viable population sizes and physiologies, streptomycin and tailocin-treated cultures colonized bean leaves at the same rate as untreated cells (Fig. 5A) and were able to cause normal symptoms of leaf spot and chlorosis at 8 days (Fig. 5B). This finding indicates that the distinct physiological states of streptomycin and tailocin persisters do not delay their ability to colonize a susceptible host. It also suggests that in the streptomycin-stressed inoculum, the large populations of high-redox nonculturable cells may not make a significant contribution to early infection, or at least not enough to speed colonization of a susceptible host.

To test the latter hypothesis, we performed a second experiment in which streptomycin and untreated cultures were adjusted to contain the same proportion of redox-active cells, regardless of intensity or culturability. Inocula adjustments were based on microscopic observations of mean RSG staining from Figure 2. In this experiment, both inocula contained 7×10^4 visibly RSG-staining cells per mL, but the untreated culture contained an estimated 450-fold greater concentration of culturable cells than the streptomycin-treated inoculum. The streptomycin-treated population started growing in the leaf much

more slowly than the untreated inoculum, with the population only increasing after a two-day lag (Fig. 5C). This further supports the hypothesis that in a physiologically heterogeneous antibiotic-stressed *Pph* population, the high-redox unculturable cells do not significantly contribute to early host colonization.

Streptomycin eradicates *Pph* persisters of tailocin

Having determined that *Pph* tailocin persisters exist in a distinct physiological state from streptomycin persisters, we next hypothesized that streptomycin could eliminate tailocin persisters and vice-versa. Antibiotic persisters often exhibit multidrug tolerance, so we also asked whether tailocin persisters could be eliminated by two other antibiotics, the bacteriostatic translational inhibitor tetracycline or the DNA replication inhibitor ciprofloxacin. Cross-survival rates of streptomycin persisters were first tested with a sequential treatment of tailocin, tetracycline, or ciprofloxacin. 30 to 70% of the streptomycin persistent population remained culturable after tetracycline or ciprofloxacin treatment, while only 1% remained culturable after tailocin treatment (Fig. 6A). Conversely, when tailocin persisters were washed and treated with tetracycline or ciprofloxacin, means of 7.2 and 11.5% remained culturable after treatment, respectively, while no colonies could be recovered after streptomycin treatment (Fig. 6B). To determine how this compares to whole population survival rates to these antibiotics, we treated stationary phase *Pph* cultures with tetracycline or ciprofloxacin alone, and measured CFU recovery at 6.5±2.3% and 0.06±0.02% of the initial population, respectively. Thus, compared to untreated *Pph* cells, tailocin persisters exhibited no survival to streptomycin, a similar survival rate to tetracycline, and a 178-fold increased rate of survival to ciprofloxacin. CCCP-treated cells were also highly multidrug tolerant, but fewer than 0.1% survived tailocin treatment (Fig. 6C).

To further check for elimination of viable unculturable cells, we concentrated and microscopically examined the streptomycin-treated tailocin persisters, and found that all cells stained with PI only or were unstained (Fig. S8). No Category 1, 2, or 4 cells were observed. To rule out the possibility of rare live cells reviving to colonize the host, bean leaves were inoculated with concentrated cultures after the combination treatment. No symptoms developed on leaves (Fig. S8), and no *Pph* colonies were recovered in leaves collected immediately after inoculation or at days 1-5. In summary, cells surviving antibiotic and CCCP treatments have a high propensity to survive treatment with other antibiotics, but are mostly eliminated by tailocin. Streptomycin treatment is highly effective at eliminating culturable tailocin persisters, while tetracycline and ciprofloxacin are less effective.

Discussion

Membrane-disrupting treatments have long shown promise for disease control and for eradication of antibiotic persisters, and there remains an enormous trove of membrane-disrupting biological compounds still to be discovered (35). Understanding the basis and management of population-level tolerance to these compounds will be important to maximize their efficacy. Here, a study of physiological heterogeneity in the model plant pathogen *Pph* demonstrated that tailocin and streptomycin treatments have vastly different physiological consequences. The small fraction of

culturable cells surviving each treatment exhibited distinct redox phenotypes, and corresponded closely with the fractions capable of causing infection. The study shows that that streptomycin and tailocin could be a potent combination treatment for sterilization of *Pph* cultures, including the elimination of viable nonculturable cells. The study also links redox states to heterogeneous persistence and virulence phenotypes, which could inform the search for associated mechanisms and markers.

Tailocins are triggered by recognition of specific lipopolysaccharides (LPS) on the target cell surface, after which the tail is driven into the membrane using energy stored in its contractile structure (36). The consequences are rapid membrane depolarization and ATP depletion, as well as transcriptional and translational arrest, from as little as one particle per target cell (37, 38). How then, can persisters survive and maintain redox activity? The finding of nondormancy would be consistent with an active mechanism of tailocin survival, and the rapid timeframe of killing suggests that this trait is expressed in a portion of the planktonic population rather than being tailocin-induced. The metabolic activity of tailocin persisters is reminiscent of conditional tolerance to the membrane-destabilizing peptide colistin in *Pseudomonas aeruginosa* and other animal pathogens. Colistin tolerance was associated with increased expression of *Pmr* proteins that modify LPS to reduce colistin affinity, meaning that only transcriptionally active cells avoid membrane destabilization (27, 28, 39). Tailocin sensitivity is also linked to the composition of LPS (40), which can vary according to changes in environment and gene expression (41). We recently found that an LPS cluster gene of unknown function affects the frequency of tailocin persisters without impacting tailocin susceptibility or host fitness (30). One hypothesis consistent with our findings would be that persistence derives from active tailocin avoidance, potentially through population heterogeneity in tailocin recognition targets or other susceptibility factors. Further molecular analysis of the persistent population will be needed to pinpoint the underlying mechanisms. The streptomycin sensitivity and ciprofloxacin tolerance we observed in tailocin persisters also echoes recent work on colistin, which was found to be much more efficient in eliminating aminoglycoside persisters than eliminating ciprofloxacin persisters in *E. coli* (10). The authors hypothesized that colistin works synergistically with aminoglycosides due to the membrane damage exerted by both treatments. Our study suggests that aminoglycoside synergy, and perhaps fluoroquinolone cross-tolerance, could be common themes of LPS destabilizing antimicrobials.

This study employed complementary methods of flow cytometry and fluorescence microscopy to profile antimicrobial-induced changes in *Pph* populations. Microscopy was useful in distinguishing intermediate viability categories, demonstrating that a third of redox-active cells were permeable to PI after streptomycin treatment. Membrane-damaged live cells were previously found to self-repair and resuscitate from VBNC populations of *Pseudomonas* and *Shewanella* sp. (42), and have been observed in nonstressed growing populations of other bacteria (43). This study indicates that DRAQ7 provides a more confident indication of fatal membrane damage for *Pseudomonas*. Streptomycin also induced a high proportion of “Category 4” cells retaining intact membranes and increased roundness but no redox signal, a state similar to one associated with persisters, VBNC cells, and newly dead cells in *E. coli* (7, 8). We hypothesize that Category 4 includes the fraction associated with streptomycin persistence in sorting experiments, which similarly lacked redox signal above that of dead cells. Tailocin reduced the proportion

of Category 4 cells in *Pph* cultures by 99.9%, consistent with its ability to target dormant cells. However, the abundance of cells in each microscopy category far exceeded the abundance of persisters, illustrating that each phenotype contains heterogeneity and that persistence levels cannot be anticipated by staining phenotype alone.

Streptomycin treatment shifted the majority of the intact *Pph* population to a state of increased RSG staining intensity. Antibiotics stimulate the production of reactive oxygen species (ROS) in bacteria (44), and antibiotic-induced increases in RSG intensity were recently associated with accumulation of ROS-protective reductases in *Campylobacter jejuni* (45). Thus we suspect that the high-redox fraction in *Pph* similarly reflects a reductase response to intracellular ROS production, although ROS-specific methods would be needed to determine this conclusively. If so, the non-culturability of this fraction could be consistent with findings that ROS avoidance is a marker of persistence and post-antibiotic culturability (46, 47), although in our study even the fraction of moderate RSG intensity was unculturable after streptomycin removal. All intact cells would be counted as live or VBNC cells using common permeability-based quantification methods (48), but the high-redox cells we observed are distinct from the VBNC cells induced by long-term starvation, reported as being dormant and persister-like (6, 7). This study demonstrates that the large live but unculturable fraction does not revive in a susceptible host or greatly contribute to short-term infection in a mixed population.

Even in the absence of antimicrobial treatment, the high-redox fraction of *Pph* did not cause symptoms on the host. In recent years it has become clear that pathogenicity on plants is often a heterogeneously expressed trait, with essential virulence factors produced in a population bistable manner in *P. syringae* and other plant pathogens (49, 50). The virulent state is associated with a suppression of genes involved in active growth processes (51, 52). This study links one avirulent *Pph* subpopulation to an increased redox signal. It is striking that five hours of exposure to a long relied-upon disease control treatment did not greatly reduce the number of intact and active cells, but rather shifted much of the population toward a non-infectious state. A more complete understanding of how antibacterial treatments affect pathogen physiology, both in the lab and field, will be essential in tailoring disease control strategies that are more effective in reduction of pathogen inoculum.

Declarations

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Conflicts of Interest

The authors declare that they have no competing interests affecting this work.

Materials And Methods

Bacterial strains, plant lines, and culture conditions

Tailocin was prepared from cultures of *P. syringae* pv. *syringae* (*Psy*) strain B728a. Experiments were performed using *P. syringae* pv. *phaseolicola* (*Pph*) strain 1448A. Cultures were grown from a single colony in King's B medium (53) at 28° C, 200 rpm shaking, unless otherwise indicated. Common bean (*Phaseolus vulgaris*) variety Kentucky Wonder (Seed Savers' Exchange, Decorah, Iowa) were grown in disposable plastic pots (8 × 6 cm and 8 cm deep) in PRO-MIX growing medium (BX, M) and maintained at 23°C with 70% relative humidity and 16 hours daylength in a Conviron growth chamber. For bean pod inoculations, plants were grown in a greenhouse (24 to 26 °C) in large pots (12 cm diameter and 12cm deep). Pods were collected from 50 to 55 day-old bean plants.

Tailocin preparation

Tailocin was prepared and quantified from supernatants of *Psy* B728a as previously described (32, 54). Overnight cultures of B728a were diluted 1:100 in King's B, grown for 3 hours at 28 °C, and tailocin production was induced by addition of mitomycin C (MP Biomedical LLC, Solon, Ohio) to a concentration of 0.5 μg mL^{-1}. After 24h induction, supernatants were collected by centrifugation. Residual live cells were killed by treating supernatant with chloroform. The aqueous phase was collected by centrifugation, then amended with NaCl and Polyethylene glycol 8000 to final concentrations of 1M and 10% w/v, respectively. After 1h incubation on ice, the supernatant mixture was centrifuged at 16,000 x g for 30 minutes at 4 °C. The resulting tailocin pellet was dissolved in 10 mM Tris (pH 7.0) and 10 mM $MgSO_4$. Residual PEG 8000 was removed by two extractions with equal volumes of chloroform. The activity of prepared tailocin was evaluated by spotting 5 μl serial dilution onto soft agar overlay plates seeded with *Pph*. Tailocin activity was expressed in activity units (AU) derived from the highest dilution factor resulting in a visible inhibition zone (55).

Minimum inhibitory concentration (MIC)

The MICs of streptomycin (MP Biomedical LLC, Solon, Ohio), tetracycline (MP Biomedical LLC, Solon, Ohio) and ciprofloxacin (Acros Organics, Fair Lawn, New Jersey) to *Pph* were determined by evaluation of turbidity using a previously described method (56) with some modifications. An overnight culture of *Pph* was diluted to an OD_{600} of 0.1 in King's B medium, and 20 μL of the cell suspension was added to 180 μL of King's B amended with antibiotics to achieve final antibiotic concentrations of 25, 12.5, 6.25, 3.12,

1.56, 0.78, 0.39, 0.19 and 0 μg mL^{-1} in 200 μL volume. Growth was assessed by measuring the OD_{600} over 20 hours using an absorbance plate reader (Bio-Tek). The MIC of each antibiotic was the lowest concentration at which no increase in turbidity was measured across at least three independent cultures. MIC for tailocin was similarly determined in activity units (AU), starting with nine 1:2 serial dilutions of the initial tailocin preparation.

Killing curve of *Pph* after treatment with streptomycin and tailocin

To prepare stationary phase cultures of *Pph*, a single colony was inoculated into 5 mL King's B broth, grown for 20h at 28 °C, diluted 1:100, and grown for 18 h (to a typical OD_{600} of 1.3) or 4 days. To prepare exponential phase cultures, a 20 h culture was diluted 1:50 in King's B medium and incubated for 2.5 h (OD_{600}= 0.15). To perform killing curve experiments, streptomycin was added to the cultures to reach a concentration of 16 μg mL^{-1} (5x MIC), followed by shaking incubation at 28 °C for 5 hours. 1 mL samples were collected prior to streptomycin addition (T=0) and hourly for 5 hours. Samples were centrifuged two minutes at 13,000 rpm and resuspended two times in sterile saline (0.8 % NaCl) and enumerated by dilution plating on King's B agar. Colonies were counted at 48h.

Tailocin killing curves were generated as previously described (30), with modifications. Log, stationary, or 4-day *Pph* cultures were diluted to OD_{600}= 0.1 in 0.8 % NaCl, and tailocin was added to a concentration of 250 AU mL^{-1}, which represents 5X MIC for *Pph*. Samples were removed before and immediately after addition of tailocin and then each hour for 4 hours. Samples were washed twice with saline and enumerated by serial dilution. Addition of the first wash was typically completed in under 3 minutes from sample collection, therefore the sample removed immediately after tailocin addition was termed t=0.05.

Microscopic cell physiology analysis of *Pph*

Staining of *Pph* with redox sensor green (RSG) and propidium iodide (PI) was performed using the BacLight RedoxSensor Green Vitality Staining Kit (with additional staining with Hoechst 33342 (both from Thermo Fischer Scientific, USA). *Pph* cultures at log and stationary phase were treated with streptomycin (5h) or tailocin (4h) as described above. Total cells were enumerated by hemocytometer count under phase contrast microscope. Culturable cells were enumerated by dilution plating, after washing the cells twice with saline (0.8% NaCl). Agarose pads (1.5%) were prepared on glass slides as previously described (57). Treated and untreated cells (10 μL) were amended with 0.1 μL RSG (1 mM), 0.1 μL PI (20 mM) and 0.15 μL Hoechst 33342 (1 mM) and incubated 10 m in the dark. 1 μL culture was placed on the middle of the agarose pad. Images were collected using a Zeiss Axio Imager M1 fluorescence microscope within 15 minutes of placement on the pad. Multichannel images were captured using FITC, rhodamine, and DAPI filter sets in Zen 2.6 (blue edition) software. For each of four independent experiments, 10 fields were imaged across at least three different slides per treatment. All cells were counted in each image, totaling a range of 1000 to 1700 cells for each treatment and timepoint in each experiment, except for the tailocin 4h timepoint, for which there were 650-700 cells in the 10 fields. Single cells were classified into five staining categories (green/blue, red/green/blue, red/blue, blue,

or unstained) by visual comparison of the same cell under three different channels and in phase contrast. Cell counts were recorded by clicking on each cell using the Cell Counter plugin in Fiji (58). Intensity and roundness of selected cells were measured using the MicrobeJ plugin in Fiji (59).

Flow Cytometry

The physiological state of streptomycin and tailocin-treated cultures were evaluated through redox and cell-integrity staining followed by flow cytometric analysis. Four treatments were selected for flow cytometry: stationary phase *Pph* culture, and stationary phase treated with streptomycin, tailocin, and CCCP as described above. Stationary phase cells were washed in saline and resuspended in saline to OD_{600}= 0.2 before treatment. After treatment, *Pph* cells were washed twice with phosphate buffered saline (PBS) and then the bacterial suspension was stained with RSG (1 mL bacterial suspension was incubated with 1 µl of RSG working solution (1 mM) and 3 µL DRAQ7 working solution (3 mM) for 30 minutes in the dark at room temperature. Stained samples were analyzed on the BD FACSAria-II at the Yale Cell Sorting Facility (Yale School of Medicine, New Haven, United States). Excitation wavelengths were 488 nm and 633 nm. Fluorescence was collected by 530/30 bandpass filter for RSG and 710/25 for DRAQ7. Data analysis was performed using BD FACSDiva 8.0.1 and FlowJo™ 10.6.2.

Fluorescence Assisted Cell Sorting (FACS) and culturability and virulence tests

During flow cytometry, the forward scatter/side scatter (FSC-A/SSC-A) dot plots of each isolate were used to define the total bacterial population. Doublets and debris were excluded via the contour and dot plots. Based on 50,000 sorted events, regions of green and red fluorescence intensity defined by stationary phase, ethanol-killed, and unstained *Pph* cells were used to define three gates associated with bright redox signal intensity (G1), medium intensity (G2), and low intensity (G3) among intact cells. Cultures were aseptically sorted into tubes until 10^7 events were collected, or for low-density gates, until the entire suspension was sorted. Collected fractions were adjusted to a final concentration 0.1 % sterile peptone buffer, a common diluent used in *Pph* enumeration (60), in a final volume of 1 mL. Isolated dots outside the polygon were not included in the analysis.

For culturing studies, sorted fractions were centrifuged at 13,000 rpm for two minutes and pellets were resuspended in 50 µL PBS. 20 µL aliquots of the suspension were serially diluted and 5 µL was spotted in triplicate on King's B agar plates. Colonies were enumerated after 48h incubation at 28 °C.

Sorted fractions were inoculated on detached bean pods according to the method of Bozkurt and Soylu (61). In brief, mature bean pods (from 50-day old plants) were collected, washed in distilled water, surface sterilized in 70% ethanol, and pierced using sterile 10 µL pipette tips. 30 µL of the 50 µL concentrated sorted fraction were placed on the wound. Inoculated pods were stored in sterile plastic containers lined with moist Whatman filter paper and incubated in a 28 °C chamber. Disease symptoms were recorded at 5 days after incubation.

Pph inoculation to bean plants

Fifteen-day-old bean plants were inoculated with untreated, streptomycin-treated, tailocin-treated, or streptomycin and tailocin-treated stationary phase cultures of *Pph*. Treated cultures were prepared using the methods and ending timepoints described for stationary phase killing curves, with streptomycin/tailocin combination treatments incubated for 4 hours. Untreated cultures were diluted in PBS to OD=0.0001, and single antibiotic-treated cultures were diluted to achieve the same concentration of culturable cells as the untreated inoculum (Fig. 6A experiment) or the same concentration of RSG-staining cells as the untreated inoculum (Fig. 6B experiment). Concentration adjustments were made based on observations from repeated prior experiment. 200 µL inoculum was infiltrated into the underside of the primary leaves of bean plants using 1 ml BD syringes. Samples from infiltrated areas were collected at 0, 1, 2, and 5 dpi using a 1 cm cork borer. Leaf discs were collected into a 1.5 ml tube containing 200 µL 10 mM $MgCl_2$ and homogenized using disposable pellet pestles (Fischer Scientific). Homogenates were serially diluted on King's B agar supplemented with 50 µg mL^{-1} nalidixic acid, to which *Pph* is genetically resistant, and CFUs were enumerated after 48h incubation at 28 °C.

Antibiotic cross-tolerance experiments

To measure cross-tolerance of streptomycin-tolerant *Pph* cultures against other antibiotics and tailocin, stationary phase *Pph* cultures were washed and resuspended to OD600= 0.1 in saline and treated with 16 µg mL^{-1} streptomycin for 5h. Cells were washed twice and resuspended in saline to remove streptomycin, then treated for 4h with tailocin (250 AU mL^{-1}), tetracycline (8 µg mL^{-1}), or ciprofloxacin (8 µg mL^{-1}) before washing again and serially diluting. Low-CFU samples were resuspended in a reduced volume of saline (50 µL) after the final wash. The same procedure was followed to determine the tolerance level of carbonyl cyanide m-chlorophenylhydrazone (CCCP)-treated or tailocin-treated stationary phase cultures to other antibiotics, with the following modifications: cultures were treated with CCCP (Sigma Aldrich) at a concentration of 100 µg mL^{-1} for 3h, or with 250 AU of tailocin for 1h.

Statistical Analysis

Differences in total and culturable cells were assessed using a Student's T-test (two-tailed distribution with two-sample, equal variance calculations). Multiple comparisons were performed with one-way ANOVA. Means were separated using Tukey's Honest Significant Difference Test at $p = 0.05$. Statistical analyses were performed in R version 4.0.3.

Data Availability

Images of fields used to generate Figure 2, Figure S2, and Supplemental Tables 1 and 2 are available in FigShare (private link for reviewers https://figshare.com/s/9bbe6f7b2fbb583c49f6).

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Figures

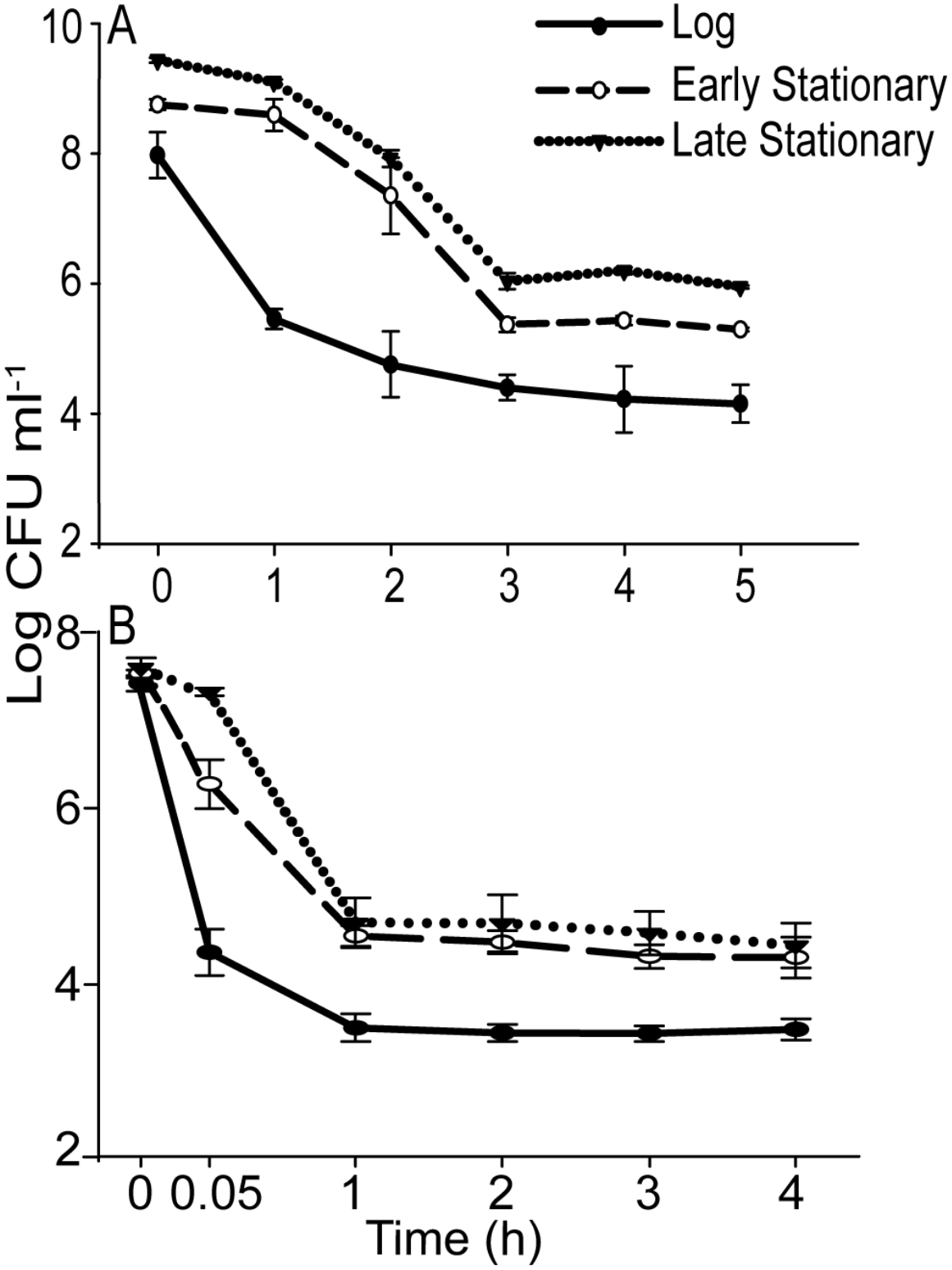

Figure 1

Comparison of persister frequencies after treatment of Pph1448a with streptomycin (A) or tailocin (B). Pph cultures were treated in log phase, early stationary phase (20 h), or late stationary phase (96h). (A) Pph cultures were treated with 5X MIC of streptomycin (16 µg mL-1), and culturable populations were enumerated hourly for 5h. (B) Cultures were treated with 5x MIC of tailocin (250 AU) after resuspension in saline to ensure a standardized tailocin ratio per target cell, and culturable populations were enumerated

immediately after tailocin addition (0.05h, or 3 minutes) and hourly. Error bars represent the standard deviation of the mean for three replicate cultures. The experiment was performed three independent times.

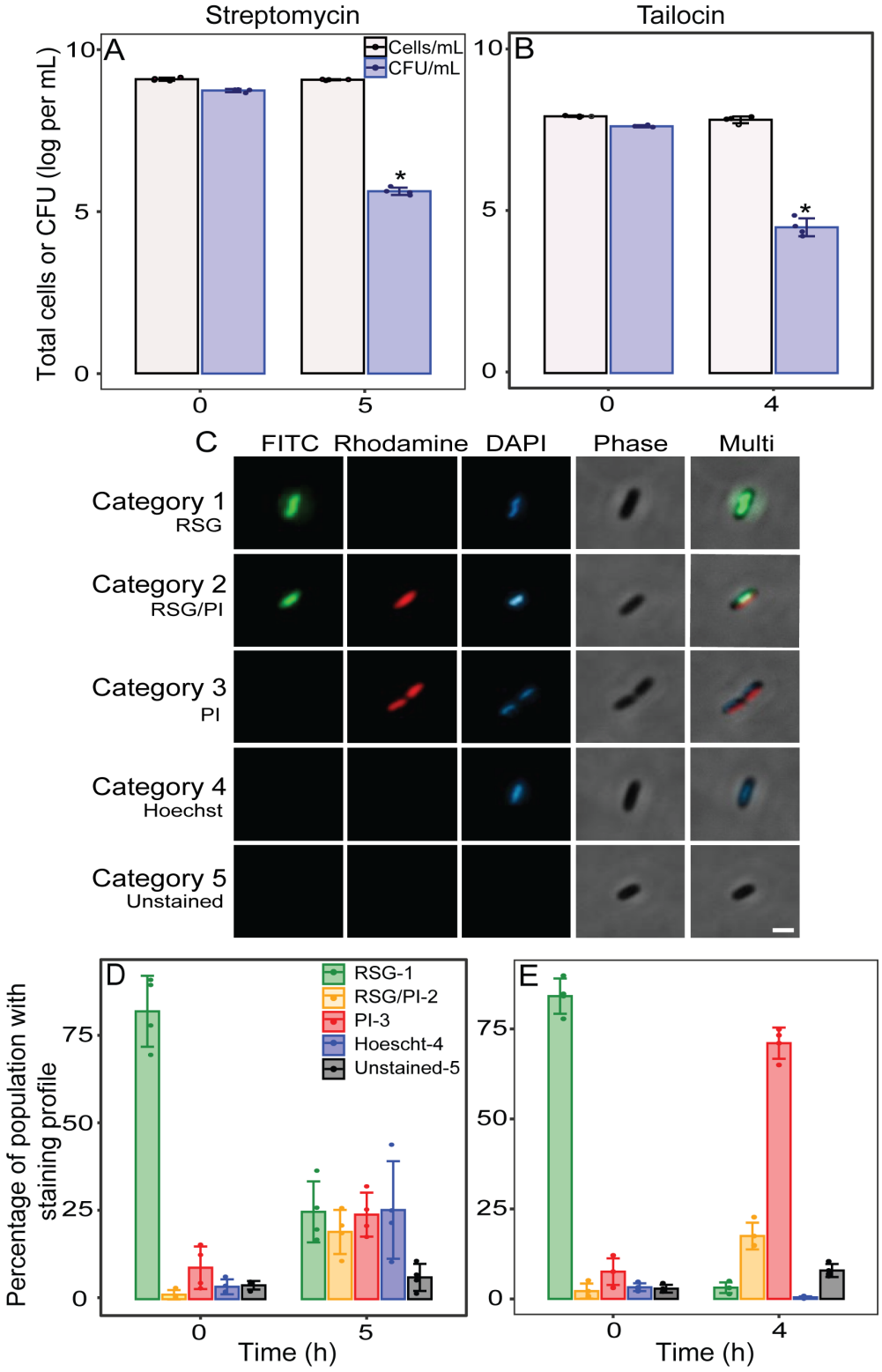

Figure 2

Distinct physiological states in stationary phase Pph cultures after treatment with streptomycin or tailocin. Cultures were treated with streptomycin (A) or tailocin (B) and enumerated by hemicytometer

(grey bars) or by dilution plating (blue bars). Asterisks represent significant difference compared to T0 ($p<10^{-6}$). Tailocin-treated cultures averaged 20% fewer cells at T4 than T0, but this was not statistically significant ($p=0.092$). (C) Representative cells depicting Pph staining categories used in physiological profiling. Cultures were stained 10m with RSG, PI, and Hoechst 33342 and imaged on an agarose pad (1.5%). Rows depict single Pph cells imaged through three fluorescent filters, in phase contrast, and combined (40x objective). Images are taken from the same image of streptomycin-treated Pph. Scale bar = 2 μm. (D, E) Proportional composition of Pph individuals in five physiological categories before and after treatment with streptomycin (D) or tailocin (E). Each datapoint represents the total proportion of all cells in a category at each culture and timepoint, counted across 10 images collected from three slides. A minimum of 650 cells were imaged per culture for tailocin at T4, and 1000 cells for all other cultures and timepoints. Categories were assigned after visual inspection of each cell under three filters and in phase contrast. The four datapoints within a bar represent values four independent experiments performed on different days. Bars represent the means and standard deviations across experiments.

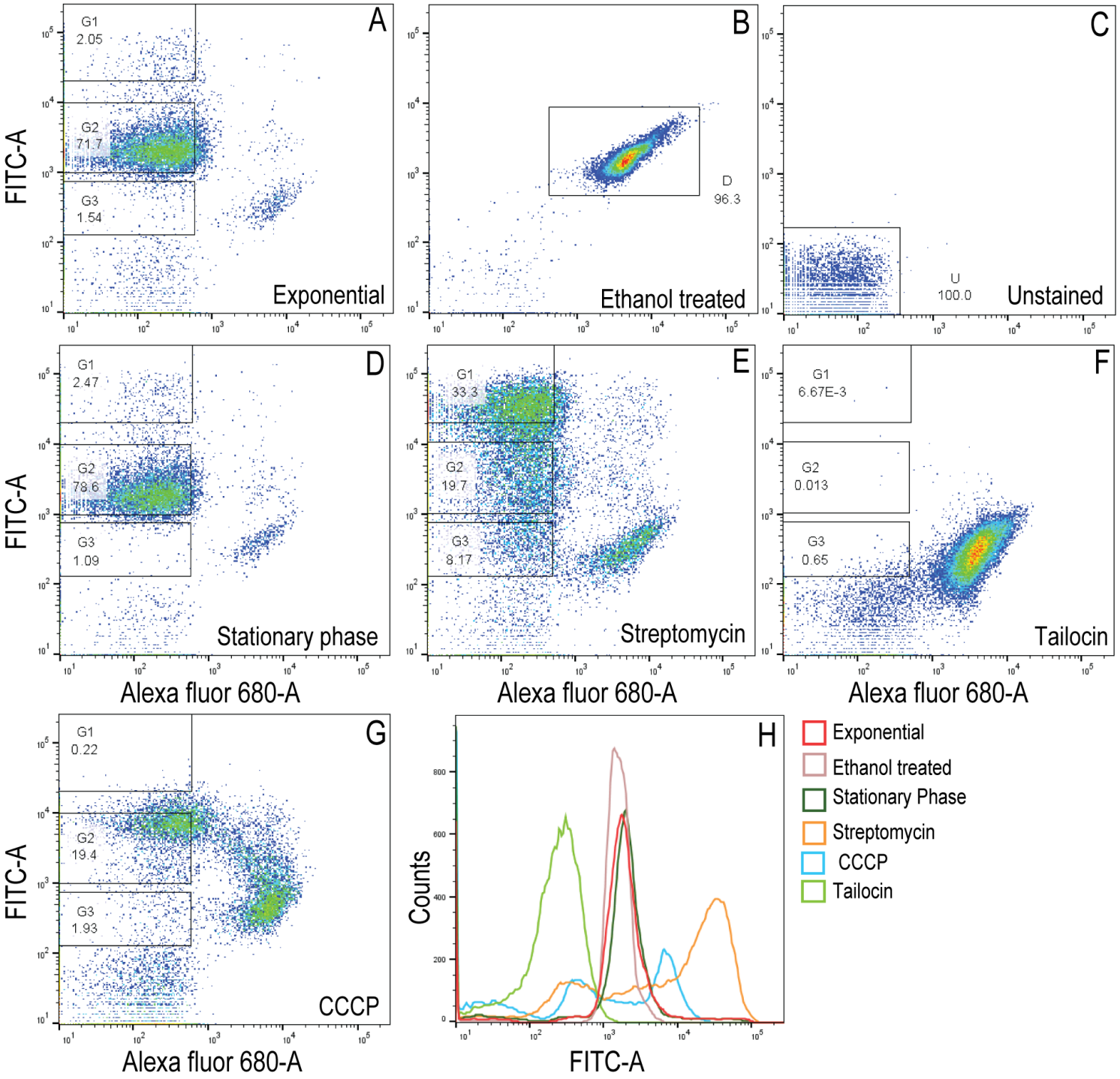

Figure 3

Flow cytometric analysis and sorting of Pph populations. (A-G) Density dot plots of RSG and DRAQ7-stained Pph cultures in log phase (A), after treatment with ethanol (B), without fluorescent stain (C), and stationary phase cultures with no treatment (D) or after treatment with streptomycin, tailocin, or CCCP (E-G). Within the DRAQ7-unstained fraction, three gates were assigned according to green fluorescence intensity above unstained cell levels, and these were designated G1-G3 (boxes in A and D-G). X and Y axes are in relative fluorescence units. Numbers in boxes represent the proportion of total sorted events that was within each gated area. (H) Histogram showing distribution of green fluorescence intensity of

populations plotted in (A-G). Figure represents experiment 1 of the two independent experiments depicted in Figure 4.

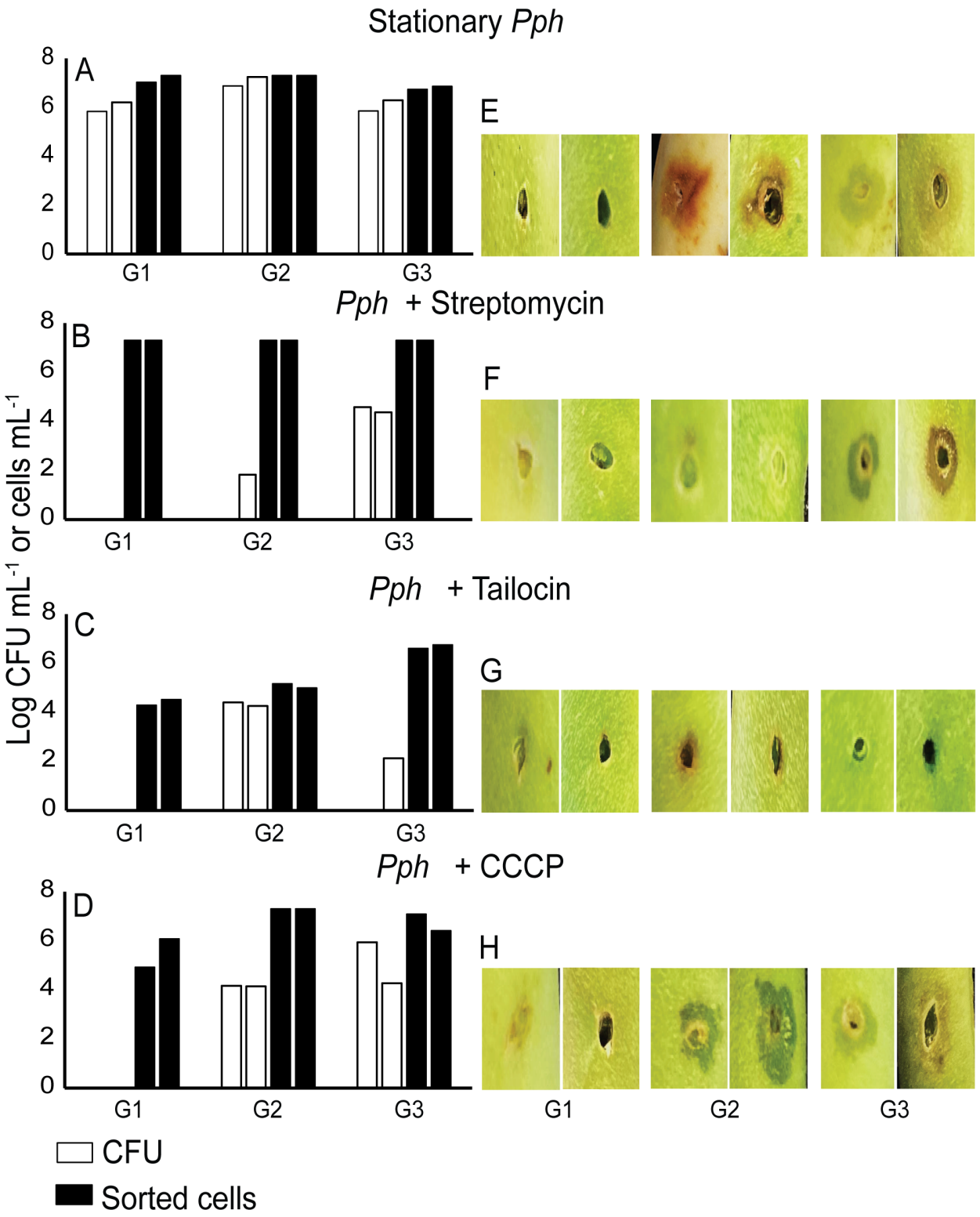

Figure 4

Culturable and infectious streptomycin and tailocin-treated cells have distinct redox staining intensities. (A to D) Culturability of cells in RSG intensity gates G1-G3. Black bars indicate total cells collected, and white bars represent CFU mL-1, enumerated from the sorted fraction. The two bars in each sorted gate

represent values from independent experiment 1 (left bar) and 2 (right bar). Samples were plated after sorting from stationary phase Pph cultures without treatment (A) or after treatment with streptomycin (B), tailocin (C), or CCCP (D). (E to H) Symptom development on bean pods after wounding and inoculation with 30 uL of the sorted fraction after 20x concentration. Images were taken 5 days after inoculation. Left and right images correspond to independent experiments 1 and 2, respectively.

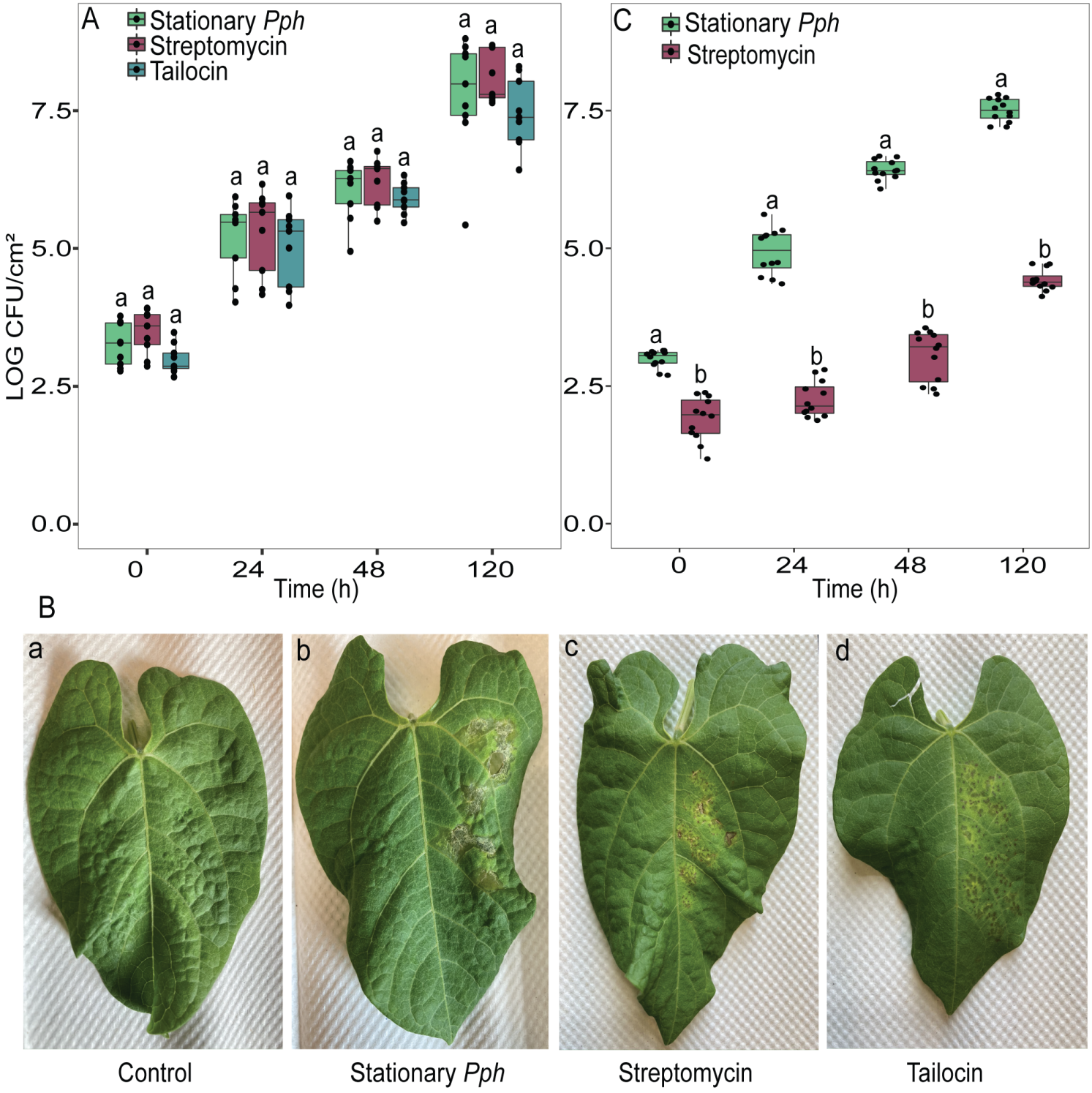

Figure 5

Colonization of host leaves by antimicrobial treated Pph. Antimicrobial-treated populations Pph colonize leaves and form symptoms at the same rate as untreated populations and form symptoms when concentration is adjusted based on culturable cells (A,B), but exhibit delayed colonization when concentration is adjusted based on RSG-staining cells (C). (A) Culturable Pph population growth in bean leaves after infiltration with untreated, streptomycin-treated, and tailocin-treated inocula that were adjusted based on CFU concentration. Inocula (200 µL) were syringe-infiltrated into primary leaves of 15-day-old bean plants, and CFUs were enumerated from leaf discs at the indicated timepoints. Dots represent combined biological replicates from two independent experiments. (B) Symptoms on bean leaves imaged 8 days after infiltration with untreated, streptomycin-treated, or tailocin-treated Pph, and water control. (C) Pph population growth in bean leaves after infiltration with untreated or streptomycin-treated inocula that were adjusted to contain equal proportions of redox-active cells, although proportions of culturable cells differed. Dots represent biological replicates from three independent experiments. Letters on the box plots denote statistical groups within timepoints ($p \leq 0.05$).

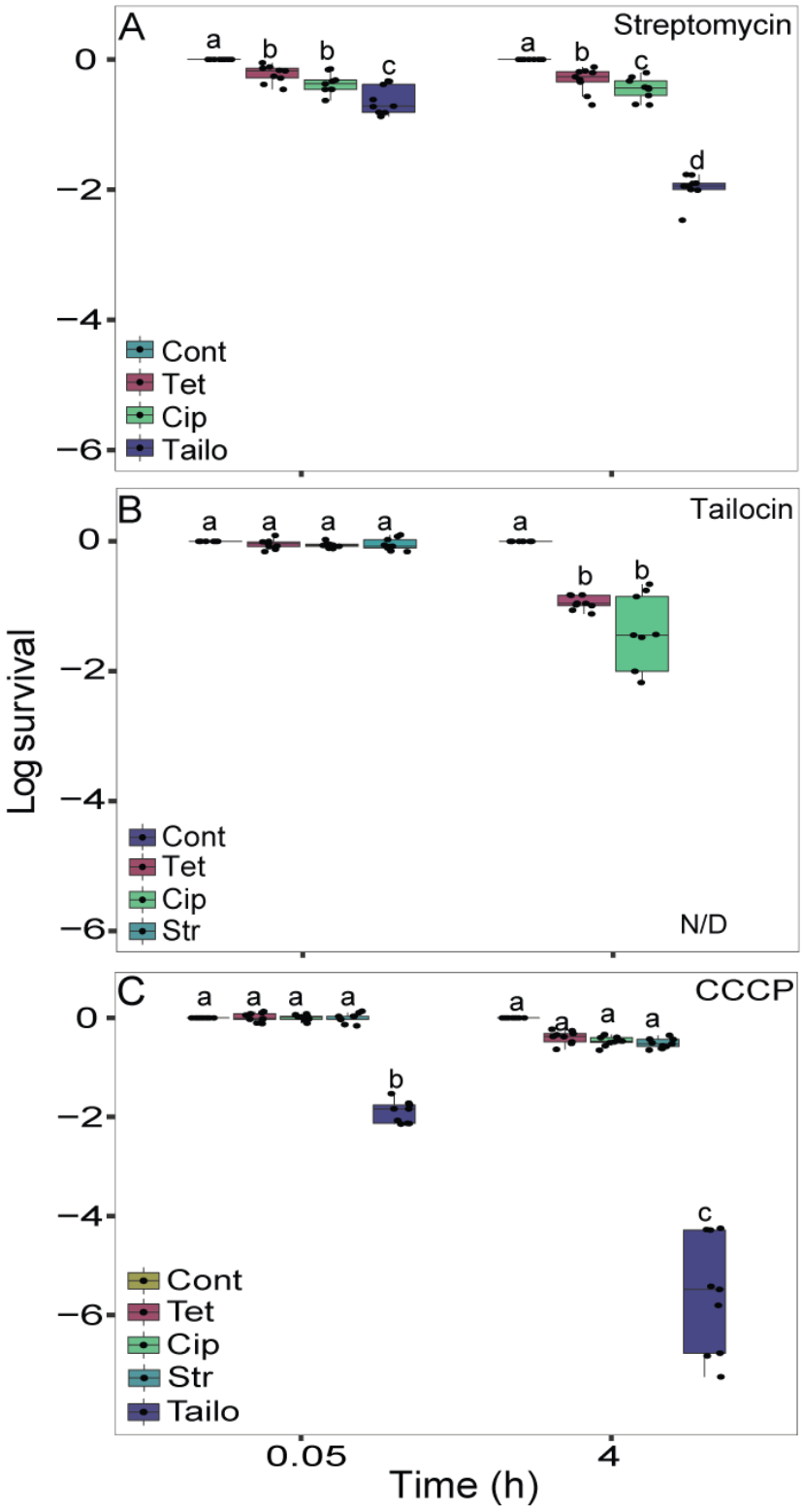

Figure 6

Tailocin effectiveness in eliminating streptomycin persisters and vice-versa. (A to C) Survival of Pph persisters of sustained treatment with streptomycin (A), tailocin (B), and CCCP (C) after washing and exposure to a second antimicrobial treatment. Stationary phase Pph were treated, washed, and then treated with 5x MIC of the secondary antibiotic (5x MIC = 7.7 µg mL-1 for both ciprofloxacin and tetracycline). Cells were washed and enumerated immediately after secondary treatment (0.05h, or 3

minutes) and at 4 hours. Dots represent nine biological replicates collected in three independent experiments on different days. Letters denote statistical groups ($p \leq 0.05$). N/D indicates a treatment from which no culturable cells were detected in any replicate.

Supplementary Files

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- TableS1.docx
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