

Bacterial Persister Cell Formation and Dormancy

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Bacterial cells may escape the effects of antibiotics without undergoing genetic change; these cells are known as persisters. Unlike resistant cells that grow in the presence of antibiotics, persister cells do not grow in the presence of antibiotics. These persister cells are a small fraction of exponentially growing cells (due to carryover from the inoculum) but become a significant fraction in the stationary phase and in biofilms (up to 1%). Critically, persister cells may be a major cause of chronic infections. The mechanism of persister cell formation is not well understood, and even the metabolic state of these cells is debated. Here, we review studies relevant to the formation of persister cells and their metabolic state and conclude that the best model for persister cells is still dormancy, with the latest mechanistic studies shedding light on how cells reach this dormant state.

PERSISTER CELLS AND INFECTION

Persister cells, those cells tolerant to antibiotics, usually comprise about 1% in the stationary state and in biofilms (1, 2). These persister cells arise due to a state of dormancy, defined here as a state in which cells are metabolically inactive. This phenotype was first described with *Staphylococcus aureus* in 1942 by Hobby et al. (3), who found that 1% of cells were not killed by penicillin and became persister cells. In 1944, Bigger (4) found that one in a million *Staphylococcus pyogenes* (*aureus*) cells was not killed by penicillin and that these surviving cells did not undergo genetic change; hence, these cells are not resistant but instead should be considered phenotypic variants that are tolerant to antibiotics. Bigger also determined that these persister cells are nongrowing by showing that penicillin did not effectively kill cells in nonnutritive medium and by showing that they had delays in regrowth in rich medium (4); hence, the first lines of evidence that these tolerant cells are dormant came from the original work with antibiotics. Bigger also recognized that penicillin was unable to clear chronic infections due to antibiotic-tolerant cells forming in patients (4) and coined the term persisters for these antibiotic-tolerant cells (4). Also, Chain and Duthie (5) confirmed in 1945 that penicillin did not completely kill *Staphylococcus* spp. until longer treatments were used and that stationary-phase cells (i.e., nongrowing cells) were nearly completely insensitive to penicillin.

Therefore, persister cells comprise a subpopulation of bacteria that become highly tolerant to antibiotics and reach this state without undergoing genetic change (6). Also, the number of persister cells depends on the growth stage. Persister cells in biofilms appear to be responsible for the recalcitrance of chronic infections, since antibiotics kill the majority of cells; however, persisters remain viable and repopulate biofilms when the level of antibiotics drops (6). Based on decades-old research, persisters are thought to be less sensitive to antibiotics because the cells are not undergoing cellular activities that antibiotics can corrupt, which results in tolerance (i.e., no growth and slow death). In contrast, resistance mechanisms arise from genetic changes that block antibiotic activity, which results in resistance; i.e., cells grow in the presence of antibiotics when they are resistant, whereas persister cells do not grow and are dormant (1). Also, this antibiotic tolerance occurs in the biofilms of members of many different genera, including *Escherichia coli* (where they are best studied), *Pseudomo-*

nas aeruginosa, *S. aureus*, *Lactobacillus acidophilus*, and *Gardnerella vaginalis* (7).

Note that the rate at which persisters form is a function of inoculum age in that older inocula have more persister cells (8). Hence, if older inocula are used, there is little difference between wild-type strains and deletion mutants that have elevated persistence (8). These authors also found, like many others, that the degree of persistence depends on the antibiotic used (8).

FORMATION OF PERSISTER CELLS VIA TOXIN-ANTITOXIN SYSTEMS

In terms of the genetic basis of persister formation, the main model for the formation of persister cells is that toxin-antitoxin (TA) pairs are primarily responsible, as they induce a state of dormancy (2, 9) that enables cells to escape the effects of antibiotics. TA systems (10) typically consist of a stable toxin (always a protein) that disrupts an essential cellular process (e.g., translation via mRNA degradation) and a labile antitoxin (either RNA or a protein) that prevents toxicity (11). RNA antitoxins are known as type I if they inhibit toxin translation as antisense RNA or type III if they inhibit toxin activity by binding the toxin protein. Type II antitoxins are proteins that inhibit toxin activity by direct protein-to-protein binding (12). Type IV protein antitoxins prevent the toxin from binding its target instead of inhibiting the toxin directly (13), and type V antitoxins are proteins that cleave the toxin mRNA specifically (14).

TA systems were first linked to persistence in 1983 (15) through ethylmethane sulfate mutagenesis of *E. coli* that led to the identification of high persistence (*hip*) mutants (16); most notably, one mutant with enhanced persistence due to the *hipA7* gain-of-function mutation was isolated. The *hipBA* locus constitutes a toxin-antitoxin locus, and the HipA toxin inactivates the translation factor EF-Tu by phosphorylating it (other substrates may also exist) (17). The structures of HipA and HipB suggest that the two substitutions of the HipA7 toxin (G22S [substitution of serine for

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glycine at position 22] and D291A) may cause it to interact poorly with the antitoxin, which would give rise to its enhanced activity that leads to increased persistence (17). Critically, the two amino acid substitutions of HipA7 render the protein nontoxic, so the mechanism by which HipA7 increases persistence is not known (except for its dependence on guanosine tetraphosphate) and is not via increased toxicity of a TA pair (16). Hence, work with HipA7, such as that showing persister cells arise stochastically (18), is not based on HipA7 acting as a toxin.

In 2004, TA systems were linked to persistence by DNA microarrays using a *hipA7* strain and ampicillin treatment to lyse nonpersister cells (19). Two percent of the genes with differential expression included those related to the YafQ/DinJ, RelE/RelB, and MazF/MazE TA systems. Overproduction of the toxin RelE led to as much as a 10,000-fold increase in persistence (19).

In 2006, TA systems were linked to persistence by DNA microarrays performed on carefully isolated dormant cells (20): using a green fluorescent protein (GFP) reporter downstream from a ribosomal promoter, metabolically inactive cells were isolated via fluorescence-activated cell sorting (FACS) based on diminished fluorescence. These dormant cells had 20-fold greater persistence to ofloxacin, so they were shown to be persisters, and DNA microarrays revealed that, compared to transcription levels in actively growing cells, these cells had the largest change in terms of elevated transcription of the toxin gene *mqsR* (20). Other TA-related genes with differential transcription included *dinJ*, *yoeB*, and *yefM*.

Only two TA pairs have been directly related to persistence in planktonic cells by their deletion; in contrast, overproducing almost any toxin increases persistence. The first work demonstrating this deletion phenotype was with the MqsR/MqsA TA system: deleting *mqsR*, as well as deleting *mqsRA*, decreased persistence (21); these results were corroborated by an independent laboratory (8). Later, the type I TA system TisB/IstR-1 was linked to persistence, since deletion of the *tisAB-istR* locus reduced persistence (22). Subsequently and confirming these two reports, it was shown that deleting multiple TA systems decreases persistence (23). Prior to the discovery of *mqsRA* and persistence, the *hipBA* TA locus was reported to be related to persistence via deletion (19); unfortunately, this result was retracted (22), as the phenotype was due to inadvertent deletion of more than just the TA loci. Also, for biofilm but not for stationary-phase planktonic cells, deletion of the gene that encodes the toxin YafQ decreased persistence to cefazolin and tobramycin 2,400-fold (24).

The proposed mechanisms by which TA systems cause persistence are linked to dormancy. For the TisB/IstR-1 system, the TisB toxin decreases the proton motive force and ATP levels, which causes the cells to become dormant (22). Cells producing the TisB toxin became persistent to several antibiotics, including ampicillin (a cell wall synthesis inhibitor that kills growing cells), ciprofloxacin (effective in killing nongrowing cells), and streptomycin (inhibits protein synthesis). For MqsR/MqsA (25–27), the increased persistence arises from the MqsR toxin cleaving most of the transcripts in the cell (its 5'-GCU cleavage site is found in all but 12 transcripts); hence, MqsR renders the cell dormant by diminishing translation. By selecting for a more-toxic MqsR variant and by utilizing DNA microarrays, it was determined that MqsR also causes persistence by diminishing the ability of the cell to respond to stress (28). Corroborating the importance of TA systems for persister cell formation, Lon protease has been shown to

be necessary for persister cell formation (23); Lon activity is required to degrade labile antitoxins for type II TA systems where a protein antitoxin inactivates the protein toxin.

Moreover, there appear to be redundant ways to form persister cells. For example, overexpression of the toxin TisB is effective for inducing persistence in the exponential phase but is not effective in the stationary phase, suggesting that there are multiple mechanisms available for *E. coli* to enter the persistent state (22). The authors also proposed that cells try to do at least two things when stressed, (i) activate genes to respond to the stress in the hope of resisting it and (ii) convert part of the population to a dormant state, a bet-hedging strategy that allows a fraction of the population to survive the stress through inactivity (22). These two different responses are important for pathogens as they face various host-related stresses (oxidants, high temperature, low pH, and membrane-acting agents) (22).

ppGpp AND PERSISTENCE

To activate TA systems, the cell must respond to stress, and it appears that this stress response is most likely propagated to TA systems through the alarmone guanosine tetraphosphate (ppGpp). ppGpp is produced via RelA and SpoT (which can also degrade ppGpp) during nutrient limitation (i.e., the stringent response) and other stresses (e.g., acid stress) and serves to change transcription due to direct interactions with RNA polymerase and by its activation of RpoS (σ^S), the stress response sigma factor for the stationary phase, and RpoE (σ^E), the stress response sigma factor for misfolded proteins in the periplasm (29). ppGpp also directly reduces DNA replication and protein synthesis (29).

Since TA systems are one of the prominent elements in models of persistence, it is germane that ppGpp was found in 1996 (30) to be required for MazF toxicity; MazF is an endonuclease toxin of the type II MazF/MazE TA system. Hence, this work set the stage for the findings that ppGpp is required for persistence. ppGpp was first definitively linked to persistence in 2003 via the HipA toxin (16). Using an *E. coli* strain with the gain-of-function mutation *hipA7* in which persistence is increased 1,000-fold, it was shown that persistence conferred by the HipA7 allele was both diminished by *relA* knockout and eliminated by *relA spoT* mutation (16). Therefore, ppGpp is required for HipA7 to increase persistence. In addition, it was shown that the increase in persistence is a result of cells transitioning to a nongrowing state more rapidly (16). The link of ppGpp to persistence was rediscovered 8 years later through a study showing that the persistence of *P. aeruginosa* also requires ppGpp (31). Also, Amato et al. (32) provided additional evidence that confirmed the role of ppGpp for persistence in *E. coli*.

THE ARGUMENT FOR DORMANCY

The stress response in bacteria is accompanied by a significantly reduced growth rate (33). It is thus probable that the increased dormancy in biofilms and the dramatically reduced growth rates of persister cells are the major reasons for the reduced susceptibility of biofilms to antibiotics (34); i.e., if antibiotics target translation and if translation is repressed by toxins such as MqsR or RelE (35), then some cells can escape the effect of the antibiotic (36).

In addition to the original work of Bigger (4) and that related to TA systems (20), Kwan et al. (37) demonstrated that persister cells are metabolically dormant by showing that cells that lack protein synthesis are tolerant to antibiotics. Recognizing that a major

route to persistence is via activation of toxins, the group mimicked a type II endonuclease toxin (e.g., MqsR) by pretreating cells with rifampin to curtail transcription and achieved nearly 100% persister cells from an initial population of 0.01% (a 10,000-fold increase in persister cells). Hence, cells that are not producing protein are persisters. Corroborating this result, the group also pretreated the cells with tetracycline, which halts translation, and again converted nearly 100% of the cells into persister cells (37). Similarly, carbonyl cyanide *m*-chlorophenyl hydrazine, which halts ATP synthesis, converted nearly 100% of the cells into persister cells. Note that these three pretreatments led to similar results with two antibiotics, ciprofloxacin (5 µg/ml) and ampicillin (100 µg/ml), and that the pretreatment only reduced the viable cell population by about one half, so the dramatic increase in persister cells was achieved by converting nearly all of the initial exponential culture into persisters. Therefore, these results demonstrated that persister cells lack protein synthesis.

The results of Kwan et al. (37) also demonstrate that stress from extracellular factors like antibiotics (that are encountered by cells in the environment) induces persistence, so cells respond via genetic circuits to increase persistence and persistence can be induced beyond the levels that occur via stochastic generation. Other examples showing that persistence increases as a result of extracellular factors include the increase in persistence due to ciprofloxacin (22), a bactericidal antibiotic shown to induce the toxin TisB at subinhibitory concentrations, and the increase due to indole (38), an interspecies (39–41) and interkingdom (42) signaling molecule.

Reduced metabolic activity has also been correlated with increased persistence through persister studies performed with metabolic regulators. PhoU is a negative regulator of phosphate metabolism in *E. coli*, and deletion of *phoU* leads to a metabolically hyperactive state with increased expression of numerous genes involved in energy production (43). While deletion of *phoU* does not affect the initial percentage of persister cells, the *phoU* mutant persisters die more rapidly in the presence of ampicillin, with 100-fold-reduced CFU/ml after 3 h in comparison to their occurrence in the wild type (43). Similarly, Zhang et al. (44) showed that deletion of *crc*, the catabolite repression protein responsible for regulating the metabolism of *P. aeruginosa* cells within biofilms, leads to increased metabolic activity throughout a mature biofilm, causing reduced ciprofloxacin tolerance. Complementation through vector expression of *crc* in a Δ *crc* host restored normal metabolic activity, thus abating the reduced tolerance (44). Therefore, low metabolic activity is the key to survival of persister cells. This indicates that the significantly reduced growth rate that accompanies the bacterial stress response (33) and is characteristic of the inner-biofilm subpopulation is the major reason for the reduced susceptibility of biofilms to antibiotics (34).

In 2004, Balaban et al. (18) used an elegant single-cell approach and microfluidics with a *hipA7* strain to investigate whether persister cells form prior to antibiotic treatment. They found that persister cells appeared prior to antibiotic treatment, so there is a fraction of persisters which are formed stochastically. Critically, these persister cells have reduced growth or no growth (18). The same group extended the single-cell approach to demonstrate that the duration of the nongrowth of persister cells is a function of the activity of the toxin of a TA system (45).

THE ARGUMENT AGAINST DORMANCY

It has become fashionable in the literature to argue that persister cells are not dormant but, instead, that the persistent state is an active response to stress (31, 46, 47). However, this line of reasoning actually supports that persister cells are dormant (4), with the only change being a better understanding of the genetic mechanism by which the cells get to the dormant state; i.e., the cells respond to stress in an active manner (via genetic circuits) only as a means to achieve dormancy, and the current line of research is determining what these circuits are. Hence, the issue of whether persisters are active or passive is really a matter of whether one chooses to analyze the cells in their response to stress or analyze the cells once a dormant state is achieved. Although the majority of cells respond actively to stress, it is only the dormant cells which demonstrate persistence.

In 2011, Nguyen et al. (31) confirmed the much-earlier work of Korch et al. (16) on the necessity of ppGpp for persistence with HipA7 by demonstrating a modest decrease of 68-fold in persistence upon deleting *relA* and *spoT* in *P. aeruginosa*. Nguyen et al. (31) also reported results similar to those of Korch et al. (16) in regard to decreased persistence using similar *E. coli relA* and *spoT* mutants. Nguyen et al. (31) argued that the necessity of ppGpp implied an active response, whereas an alternative interpretation of their results is that ppGpp is required to activate a cell response that leads to arrested growth. In *P. aeruginosa*, Nguyen et al. (31) found that the reduction of 4-hydroxy-2-alkylquinoline and the production of catalase and superoxide dismutase were important for the ppGpp effect. What was not considered was the effect of the simulated stringent response on TA systems in *P. aeruginosa* and their effect on dormancy.

Orman and Brynildsen (47) utilized FACS in an attempt to differentiate nondormant cells that were actively dividing and metabolically active from those dormant cells that were not and to relate this “dormancy status” to persistence. To accomplish this, they constructed a chromosomally integrated T5 promoter (T5p)-mCherry (red fluorescent) mutant under the control of a strong isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible promoter in order to detect whether or not cells were actively dividing/reproducing. Also, to differentiate cells that were metabolically active from those that were not, they added RedoxSensor green prior to FACS. They concluded from their flow cytometry data that nongrowing (red) cells are more likely to be persisters than growing (nonred) cells but that persisters can be found in the normally dividing subpopulation. They interpreted this to mean that persistence is far more complex than dormancy and that their data point to additional characteristics needed to define the persister phenotype. However, to carry out their assays, they inoculated stationary-phase cells of *E. coli* incubated in LB medium at 37°C for 24 h into LB medium for 2.5 h prior to the FACS and persister cell assays, with the assumption that this resulted in all cells being in the exponential phase. However, Jøers et al. (48) demonstrated that stationary-phase cells of *E. coli* grown in LB medium at 37°C, when used as the inoculum, do not rapidly resuscitate and can lead to 5% of the culture having persister cells after 2.5 h at 37°C. Therefore, instead of analyzing only exponential-phase cells, Orman and Brynildsen (47) very likely carried over high numbers of dormant persister cells into their FACS and persister cell assays. While both Keren et al. (61) and Orman and Brynildsen (47) demonstrated that the ability of a

normally replicating cell to form a persister is lost after continuous exponential-phase propagation, Orman and Brynildsen (47) did not use these types of cells in the above-described FACS assays. Therefore, preexisting persister cells would be expected in their assays at time zero. Also, out of 500,000 cells that were subjected to FACS, their overall conclusion that dormancy is not necessary for persistence was based on their finding that out of approximately 100 persister cells, only approximately 20 of them sorted as metabolically active or actively growing; i.e., the vast majority (80 of them) were metabolically inactive. Given the inherent inaccuracy of FACS (they showed that the error with their FACS method was 0.2%, or 1,000 cells) and the fact that no explanation of how the boundaries between red and nonred and green and nongreen in the FACS were established, it seems very possible that if the green fluorescence boundary had been increased and/or the red fluorescence boundary decreased, 100% of the persister cells would have been characterized as nondividing and nonmetabolically active and thus classified as dormant. Hence, their claim that dormancy is not necessary or sufficient for persistence is suspect.

The study of Hofsteenge et al. (49) found that environmental isolates of nonpathogenic *E. coli* have different tolerances to different antibiotics. Based on this observation, they concluded that there may be distinct physiological states of dormancy. However, this conclusion cannot be directly drawn from these results without demonstrating that the different antibiotic-tolerant populations do in fact exhibit differing states of dormancy. Without this important analysis, it seems more likely that different tolerance levels to antibiotics could result from varying levels of induced persistence, since antibiotics are known to affect persistence (22, 37).

It has been argued that the study of Wakamoto et al. (50) with *Mycobacterium smegmatis* implies that persister cells are not dormant. This study showed that cells surviving lethal treatment with the prodrug isoniazid were metabolically active. However, isoniazid requires activation by the catalase KatG, so the cells tolerant to isoniazid were simply cells with low levels of KatG activity. In effect, these tolerant cells were never exposed to a lethal antibiotic treatment because the isoniazid remained inactive. Hence, the metabolic activity observed for cells surviving isoniazid is not indicative of metabolic activity in persister cells and this report is a special case of a prodrug requiring activation. Therefore, there is little evidence indicating that persister cells are not dormant but a wealth of evidence indicating that persister cells are dormant.

PREVENTING PERSISTENCE AND WAKING PERSISTERS

There has been some success in killing persister cells by adding glycolysis intermediates (e.g., pyruvate) which serve to generate a proton motive force that makes the cells more susceptible to aminoglycosides (51); note that these authors did not show that these compounds revert persister cells. These results suggest that persister cells are primed for more-active metabolism, since providing glycolysis intermediates stimulates metabolic activity. Also, the fact that TA systems are closely related to persistence (2, 9) and many if not all slow metabolism due to free toxins (e.g., endoribonucleases) argues that if toxin activity could be controlled, persistence might be controlled.

Since persister cells are likely dormant, it makes sense to try to wake them to make them susceptible to antibiotic treatments. By screening 6,800 chemicals in a random chemical library, 3-[4-(4-methoxyphenyl)piperazin-1-yl]piperidin-4-yl biphenyl-4-carboxylate was identified as a compound that

wakes persister cells at 25 μ M (52). However, the mechanism was not determined. Also, Pan et al. (53) found a new use for brominated furanones that have been studied for inhibiting quorum sensing by finding that (*Z*)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5*H*)-one wakes *P. aeruginosa* planktonic and biofilm persister cells. They also determined that the compound is effective with mucoid *P. aeruginosa* (54). In addition to the limited success using chemical compounds to wake persisters, there has also been some success using Trp-/Arg-containing antimicrobial peptides to kill persisters (55). Antimicrobial peptides do not function like traditional antibiotics, which disrupt cellular processes, but rather act by directly disrupting cell structure, with activity against bacteria, viruses, and fungi (56). In summary, little is known about how persister cells awakened from a dormant state to become susceptible to antibiotics, and few methods have been devised to kill persisters.

PERSPECTIVES

Given the strong link between ppGpp, TA systems, and persistence, it would be informative to investigate whether changes in ppGpp lead to direct changes in the transcription of TA systems and thereby activate toxins (when toxins are studied with physiologically relevant copy numbers). Also, it is important to demonstrate how external stress results in changes in ppGpp concentrations. Clearly, overproduction of most toxins increases persistence, but how physiological levels of toxins induce persistence is not yet clear and needs to be addressed. Since TA systems control other TA systems to form a cascade related to persistence (14, 57) and both toxins (26, 58) and antitoxins (59, 60) are global regulators, TA systems form an intricate part of how the cell responds to stress. Clearly what is needed is a way to keep toxins inactive and antitoxins active, but given the large number of TA systems in many strains, this is a formidable challenge. With an improved understanding of how persister cells form, we may be in a better position to wake them and make them more susceptible to antibiotics.

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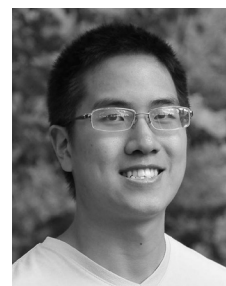
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