

## Persister cells and tolerance to antimicrobials

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### Abstract

Bacterial populations produce persister cells that neither grow nor die in the presence of microbicidal antibiotics. Persisters are largely responsible for high levels of biofilm tolerance to antimicrobials, but virtually nothing was known about their biology. Tolerance of *Escherichia coli* to ampicillin and ofloxacin was tested at different growth stages to gain insight into the nature of persisters. The number of persisters did not change in lag or early exponential phase, and increased dramatically in mid-exponential phase. Similar dynamics were observed with *Pseudomonas aeruginosa* (ofloxacin) and *Staphylococcus aureus* (ciprofloxacin and penicillin). This shows that production of persisters depends on growth stage. Maintaining a culture of *E. coli* at early exponential phase by reinoculation eliminated persisters. This suggests that persisters are not at a particular stage in the cell cycle, neither are they defective cells nor cells created in response to antibiotics. Our data indicate that persisters are specialized survivor cells.

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**Keywords:** Antimicrobial tolerance; Persisters; *Escherichia coli*; *Pseudomonas aeruginosa*; *Staphylococcus aureus*

### 1. Introduction

Bigger [3] was the first to note that a culture of growing bacteria cannot be ‘sterilized’ by penicillin and named the surviving cells ‘persisters’. Little is known about persisters (see [11,12] for a review), no doubt due to technical difficulties of working with a small fraction of cells (typically  $10^{-6}$ – $10^{-4}$  of the population) expressing a temporary phenotype of uncertain functional significance. However, we recently discovered that persisters represent the ‘missing component’ of biofilm resistance to killing by microbicidal agents [6,12,20]. Biofilms are responsible for the majority of recalcitrant infections [1,7,12] and are formed by most bacterial pathogens. The problem of biofilm tolerance now effectively shifts towards understanding the nature of persisters. It is important to emphasize that neither persisters nor biofilms are resistant to antibiotics [3,14,20,22], and do not exhibit an increased MIC (minimal inhibitory concentration) which normally results from expression of specific resistance mechanisms. Persisters, and biofilms that contain them, exhibit tolerance, which means that cells do not

grow in the presence of antibiotics, but do not die either. This ability to avoid killing is the key feature of persisters. We also found that biofilms are not unique in producing large persister populations. Stationary phase cultures of *Pseudomonas aeruginosa* produce persisters at high levels ( $10^{-3}$ – $10^{-2}$ ) and exhibit tolerance comparable to that found in biofilms [6,20]. Similarly, stationary-state cultures of *Staphylococcus aureus* and *Escherichia coli* produce large populations of persisters. This means that the mechanism of persistence can be conveniently studied in liquid culture.

The only mutants known to increase persistence to unrelated antibiotics were described in a series of papers by Moyed and coworkers [4,5,14–16,19]. A mutagenized *E. coli* culture was selected for increased survival in the presence of ampicillin, but all mutants that showed an increased ampicillin MIC were discarded. In this way, only mutants that produced higher levels of persisters were saved. Mutations in either *hipA* or *hipB* genes of the *hipAB* locus were found to increase the rate of persister production from  $10^{-6}$  to  $10^{-3}$ . Apart from ampicillin, *hip* cells showed increased tolerance to other cell wall acting antibiotics, to lethal heat shock and to DNA damaging conditions. We additionally find that *hipA* has increased tolerance to aminoglycosides (tobramycin and kanamycin). The *hip* mutations are point amino acid substitutions

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(T.M. Hill, personal communication), and deletion of the *hipAB* locus has no apparent phenotype [4]. The mechanism by which Hip proteins affect persister production is not known. HipB forms a complex with HipA. HipB is a DNA-binding protein and apparently a regulator. Null mutations in *hipB* could not be obtained, suggesting that free HipA prevents cells from growing.

In this paper, we have shown that similarly to *E. coli*, stationary phase cultures of *S. aureus* and *P. aeruginosa* produce large populations of persisters. We have also examined the dynamics of persister formation, and our results suggest that persisters are specialized survivor cells the production of which depends on growth stage.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

The strains used were *E. coli* K12 (*E. coli* genetic stock center, CGSC4401), and two K12 derivatives HM21 (AT984 *dapA zde-264::Tn10*) and HM22 (AT984 *dapA zde-264::Tn10 hipA7*) [14], *S. aureus* DB (ATCC 55585), and *P. aeruginosa* PAO1.

Luria–Bertani (LB) broth and LB agar plates were used to culture *E. coli*, tryptic soy broth (TSB) and agar (TSA) were used for *S. aureus*, and Mueller Hinton broth (MHB) and agar (MHA) were used for *P. aeruginosa*. For the HM21 and HM22 strains the medium was supplemented with 25  $\mu\text{g ml}^{-1}$  diaminopimelic acid (DAP). Overnight cultures of *E. coli* and *P. aeruginosa* were made by diluting thawed cells from an 8% DMSO (dimethyl sulfoxide) stock ( $-80^{\circ}\text{C}$ ) 1:1000 and cultured with aeration for 16–20 h. *S. aureus* cells from a 15% glycerol stock were streaked on a plate and a colony was picked and suspended in 1 ml TSA and cultured with aeration at  $37^{\circ}\text{C}$  for 16–20 h.

### 2.2. Time-dependent killing

An overnight culture of cells was diluted 1:1000 in 20 ml LB in a 125 ml baffled flask and cultured on a shaker (250 rpm) at  $37^{\circ}\text{C}$  for 3 h, thereby reaching exponential phase. 1 ml of cells was challenged in culture tubes (17 $\times$ 100 mm) by adding the appropriate antibiotics, the tubes were incubated on a shaker (250 rpm) at  $37^{\circ}\text{C}$  for 3 h. At the designated time points a sample was removed, diluted and 10  $\mu\text{l}$  was spotted on an LB agar plate for determination of colony-forming units.

### 2.3. Heritability of persistence

An overnight culture of K12 was diluted 1:1000 in 1 ml LB in culture tubes (17 $\times$ 100 mm) and cultured on a shaker (250 rpm) at  $37^{\circ}\text{C}$  for 2.5 h. Ampicillin was added to a final concentration of 100  $\mu\text{g ml}^{-1}$ , and incubated with

aeration at  $37^{\circ}\text{C}$  for 3 h, causing lysis of a subset of the population. Surviving cells were pelleted (10 000 $\times g$ , 5 min,  $10^{\circ}\text{C}$ ), and resuspended in 25 ml LB. The cells were cultured with aeration at  $37^{\circ}\text{C}$  for 16–24 h and tested for resistance to killing by ampicillin as described above. The procedure was repeated four times.

### 2.4. Growth-state dependence of persistence

Overnight cultures of the tested organisms were diluted 1:1000 into 20 ml fresh media in 125 ml baffled flasks and cultured on a shaker (250 rpm) at  $37^{\circ}\text{C}$ . At designated time points a sample was removed from each flask. 0.5 ml of cells was mixed in culture tubes (17 $\times$ 100 mm) with 0.5 ml medium +2 $\times$  drug concentration. The tubes were incubated on a shaker (250 rpm) at  $37^{\circ}\text{C}$  for 3 h. Before and after the antibiotic challenge a sample was diluted and spotted on an LB agar plate for cell count. When challenged with ofloxacin the remaining cells were washed once (10 000 $\times g$ , 1 min) with fresh medium to minimize carry-over effects. In some cases 100  $\mu\text{l}$  of the remaining cells was spread on an LB plate to increase sensitivity by lowering the limit of detection.

### 2.5. Eliminating persisters

An overnight culture of HM21 or HM22 was diluted 1:1000 into 20 ml fresh media in 125 ml baffled flasks and cultured on a shaker (250 rpm) at  $37^{\circ}\text{C}$  for 2 h and 40 min for HM21 and 4 h for HM22. At designated time points cells were diluted 1:50 in fresh medium (same volumes as above) and the diluted cultures were returned to the shaker. The dilution/growth cycle was repeated twice for the wild-type (HM21) and three times for the *hipA7* mutant. We measured the number of persisters before each dilution, as described above. To increase sensitivity, in some cases, 100  $\mu\text{l}$  of cells was spread on a plate or 1 ml of cells was filtered (Nalgene, 150 ml analytical filters) and the filter was placed on a plate. Cultures challenged with ofloxacin were washed once before diluting.

## 3. Results

### 3.1. Tolerance of the *hipA7* mutant

The *hipA7* mutant has higher tolerance to diverse antibiotics. The *hipA7* mutation leads to a 10–10 000-fold higher level of persisters (Fig. 1), in accordance with previous observations [4,8,14,21,22]. Importantly, the MIC of the *hipA7* strain is not changed (unpublished data), suggesting that higher tolerance is not due to expression of a resistance mechanism. Cells of exponentially grown *E. coli* HM21 and HM22 (*hipA7*) were treated with ampicillin (Fig. 1A) or ofloxacin (Fig. 1B) for 3 h. The cell numbers at the designated time points were determined

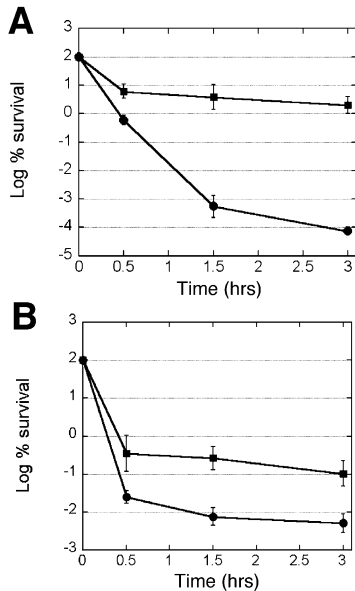


Fig. 1. Time-dependent killing of *hipA7 E. coli* by antibiotics. An exponential phase culture of *E. coli* wild-type (HM21, squares) and *hipA7* (HM22, circles) was treated with ampicillin ( $100 \mu\text{g ml}^{-1}$ ) (A) or ofloxacin ( $5 \mu\text{g ml}^{-1}$ ) (B) for 3 h. The values are averages of three replicates and error bars indicate the standard deviation.

by colony counting. The typical biphasic killing pattern was observed with a fraction of the population, the persisters, not dying even after extended incubation times in the presence of antibiotics.

### 3.2. Persisters are not mutants

It was reported in the literature that persisters are an epigenetic variant of the wild-type and their increased survival was not transferred to their progeny [3,14,22]. However, quantitative data to support this notion have not been published, and we decided to revisit this important question.

Cells of exponentially grown *E. coli* K12 were treated with ampicillin for 3 h, which led to a visibly strong lysis of the culture. A time-dependent experiment (Fig. 2)

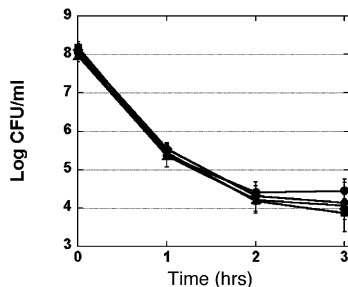


Fig. 2. A test for persister heritability. Exponential phase cells of *E. coli* K12 were challenged with ampicillin at  $100 \mu\text{g ml}^{-1}$ . Cells were then washed and cultured overnight in fresh medium, and exposed to antibiotic again. The procedure was repeated four times. Day1, circles; day 2, squares; day 3, diamonds; day 4, triangles. The values are averages of three replicates and the error bars indicate the standard deviation.

showed a typical biphasic killing with a fraction of persisters. After the 3 h incubation, remaining cells were pelleted, washed, inoculated in fresh medium and cultured overnight. This new population derived from persister cells was tested for survival to ampicillin treatment. The time-dependent killing showed essentially the same pattern as for the original population, indicating that persistence was not heritable, at least under the conditions of this experiment. Two additional cycles repeating this procedure similarly failed to enrich in persisters (Fig. 2).

### 3.3. Dependence of persister formation on growth stage

An overnight culture of either the wild-type or *hipA7* mutant was diluted 1:1000 and cultured at  $37^\circ\text{C}$  with aeration. At the designated time points (Fig. 3), samples were removed to measure the ratio of persisters. At high antibiotic concentration the bulk of cells died rapidly, leaving surviving persisters. Further increase in the level of the drug had little effect on cell death (unpublished data). In order to determine the level of persisters in a growing culture, we therefore took samples and exposed them to an antibiotic at a concentration within the plateau (where the persister fraction does not change by increasing drug concentration). Using this method the level of persisters was determined along the growth stages, from lag to stationary phase. We have chosen two antibiotics with different targets – ampicillin and ofloxacin, a  $\beta$ -lactam and a fluoroquinolone, respectively.

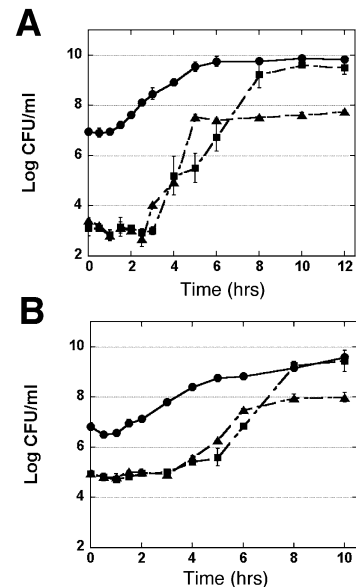


Fig. 3. Growth-stage dependence of persister formation in *E. coli*. An overnight culture of *E. coli* HM21 (A) or HM22 (B) was diluted 1:1000 and cultured with aeration at  $37^\circ\text{C}$ . At the designated time points a sample was treated with ampicillin ( $100 \mu\text{g ml}^{-1}$ ) or ofloxacin ( $5 \mu\text{g ml}^{-1}$ ) for 3 h. Circles, cell count before challenge; squares, cell count after ampicillin challenge; triangles, cell count after ofloxacin challenge. The values are averages of three replicates and the error bars indicate the standard deviation.

The growth and killing pattern of the wild-type is shown in Fig. 3A. The level of persisters was constant during lag and early exponential phases. In some experiments we observed a slight decrease in the level of persisters during the lag phase (data not shown). The number of persisters started to increase only in early to mid-exponential phase; there was a sharp increase after 2.5 h (ofloxacin) or 3 h (ampicillin). By that time the total population size increased by about 100–150-fold. There was a time period ( $\sim 1.5$ –2 h) where the population was rapidly growing but there was no increase in the number of persisters. The generation of persisters starts abruptly at a relatively high rate. The doubling time for the population was 27.5 min. The amount of persisters generated doubled every 15.4 min (ampicillin) and 10.0 min (ofloxacin). Generation of persisters reached a plateau in late exponential phase with ofloxacin. In the case of ampicillin, the level of persisters increased in the rapidly growing culture as well. Upon reaching stationary state, the entire culture became tolerant since ampicillin does not kill non-growing cells.

The dynamics of persister formation in the *hipA7* mutant was very similar to that of the wild-type (Fig. 3B). Their number was constant in the lag and early exponential phases, followed by a sharp increase in mid-exponential phase. The number of persisters in lag and early exponential phases was about a 100-fold higher for the *hipA7* mutant as compared to the wild-type, in agreement with previous observations [8,14,21]. In the case of ampicillin treatment the rate of persister generation was similar for wild-type and the *hipA7* mutant (doubling every 15 and between 15 and 18 min, respectively). For ofloxacin treatment the wild-type generated persisters at a higher rate (doubling every 10 min relative to 21 min for the *hipA7*). For ofloxacin, which can kill non-growing cells, the persister fraction in stationary state was about 1% and was similar for the wild-type and the *hipA7* mutant.

### 3.4. Eliminating persisters

At early stages of growth, the level of persisters was low and did not increase. It is possible that this reflects a steady state, where some persisters are formed and some are lost. It was also possible that persisters are left over from stationary state, and are neither produced nor lost in lag and early exponential phase. If this were the case, repeated reinoculation of early exponential culture will result in a loss of persisters.

An overnight culture was diluted 1:1000 in 20 ml LB, cultured with aeration at 37°C for 4 h (*hipA7*) or 2 h and 40 min (wild-type); after reaching mid-exponential phase it was diluted 1:50 into fresh medium and cultured again under similar conditions. The dilution/growth cycle was repeated three times for *hipA7* and twice for the wild-type (Fig. 4). The time between dilutions was optimized such that the total cell number was kept approximately constant. The number of persisters in the wild-type was

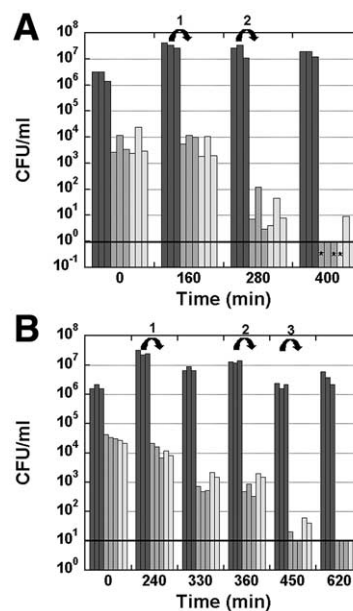


Fig. 4. Elimination of persisters in *E. coli*. Exponential phase cells of *E. coli* HM21 (A) or HM22 (B) were sequentially grown, diluted and re-inoculated at the indicated time points (arrows). The numbers of cells and persisters were determined after each growth cycle. Dark gray, cell count; medium gray, cell count after ampicillin ( $100 \mu\text{g ml}^{-1}$ ) challenge; light gray, cell count after ofloxacin ( $5 \mu\text{g ml}^{-1}$ ) challenge. The horizontal line represents the limit of detection; stars in the bars indicate that the actual cell number was below the limit of detection. The three bars represent three replicates.

reduced from  $10^3$ – $10^4$  CFU ml $^{-1}$  to  $< 1$ – $10$  CFU ml $^{-1}$  (the limit of detection was 1 CFU ml $^{-1}$ ). The size of the population in which the level of persisters was determined was kept constant, so that the loss of persisters could not be attributed to simple reduction in the overall cell numbers. In a similar experiment with *hipA7* (Fig. 4B), the persister fraction was completely eliminated (limit of detection was 10 CFU ml $^{-1}$ ) with three 1:50 dilution/growth cycles. These results indicate that persisters are not produced in lag and early exponential growth phase.

### 3.5. Persister formation in *P. aeruginosa* and *S. aureus*

We were interested in learning whether the dynamics of persister formation in other bacteria were similar to that of *E. coli*. An overnight culture of either *P. aeruginosa* or *S. aureus* was diluted 1:1000 and cultured at 37°C with aeration. At designated time points samples were removed to measure the ratio of persisters after treatment with ofloxacin (*P. aeruginosa*) or ciprofloxacin and penicillin (*S. aureus*). The overall dynamics were very similar to those observed for *E. coli* (Fig. 5), with a low level of persisters in lag and early exponential phases, followed by a sharp increase in early to mid-exponential phase, and leveling off in late exponential or early stationary phase. For both organisms, as was the case for *E. coli*, the rate of persister production was higher than the overall

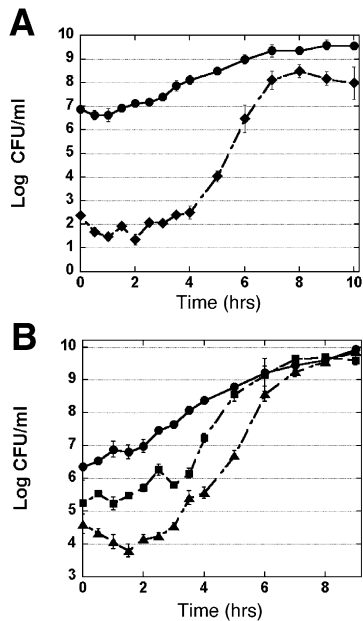


Fig. 5. Growth-stage dependence of persister formation in *P. aeruginosa* and *S. aureus*. An overnight culture of *P. aeruginosa* (A) or *S. aureus* (B), was diluted 1:1000 and cultured with aeration at 37°C. At designated time points a sample was challenged for 3 h with ofloxacin (5 µg ml<sup>-1</sup>) in the case of *P. aeruginosa* or ciprofloxacin (10 µg ml<sup>-1</sup>) and penicillin (1 µg ml<sup>-1</sup>) in the case of *S. aureus*. Circles, cell count before challenge; diamonds, cell count after ofloxacin challenge; triangles, cell count after ciprofloxacin challenge; squares, cell count after penicillin challenge. The values are an average of three replicates and error bars indicate the standard deviation.

growth rate. *P. aeruginosa* had a doubling time of 39 min while the amount of persisters generated doubled in 9.5 min. *S. aureus* had a doubling time of 30 min and the persisters generated doubled in 14 min for ciprofloxacin and 11 min for penicillin. In all cases it appeared that there was a time period, in early exponential phase, where the population was growing while the number of persisters remained constant, as was the case for *E. coli*.

#### 4. Discussion

Killing by microbicidal antibiotics is typically biphasic for a wide range of species and compounds. The presence of a residual fraction of cells that die slowly or not at all has been largely ignored, in part due to the definition of MBC-death of  $\geq 3$  log of cells [9,13]. In most cases studied, persisters will fall beyond this threshold and are not reported. We found that these residually surviving cells are largely responsible for the high levels of tolerance of biofilms to microbicidal antimicrobials such as fluoroquinolones that can kill slow-growing cells. We also found that the proportion of persisters is actually higher in a stationary phase population, and stationary phase cultures of *P. aeruginosa* are in fact more resistant to killing by antibiotics than biofilms [20]. We have made similar ob-

servations with biofilm vs. stationary populations of *E. coli* and *S. aureus* (unpublished data). These observations suggest that biofilm survival is based on the presence of persisters, and not on expression of possible biofilm-specific resistance mechanisms. Considering that biofilms are responsible for over 60% of all human infections, the study of persisters becomes highly significant.

A number of genes/mutations have been reported to affect tolerance of bacterial cells to unrelated antibiotics (*vncS*, *sulA*, *marA*, *hipA* – see [11] for a review). Findings with *vncS* and *sulA* have not been substantiated, and *hipAB* remains the only known locus that affects tolerance without changing MIC. This means that antibiotics normally reach and affect their targets in *hip* cells. *hipA7* produces 10–10 000 times more persisters than the wild-type. Presumably, antibiotics normally act against their targets in persisters as well, but unlike regular cells, they do not die. The reason for this tolerance remains unknown. The suggestion that persisters are simply non-growing [3,19] does not explain their phenotype. Indeed, fluoroquinolones kill non-growing cells, but leave persisters intact. It is possible, however, that persisters are cells in a state of deep dormancy (see [18]).

We have examined the dynamics of persister formation both in *hip* and wild-type populations. Our observations show that persister production exhibits a sharp increase at mid to late-exponential phase in *E. coli*, *P. aeruginosa*, and *S. aureus*, resembling a quorum-sensing type pattern [2,10,17]. Repeated reinoculation of a culture to maintain it in an early exponential state leads to elimination of persisters in *E. coli* wild-type and *hip* strains. Since persisters are not formed in early exponential phase, their initial presence at this stage is due apparently to a carry-over of persisters from the stationary state inoculum. The absence of persister formation at early exponential phase allows us to eliminate a number of possibilities regarding the nature of these cells. Moyed suggested that persisters represent a particular stage in the cell cycle [19]. Since early exponential cells undergo a cell cycle but do not produce persisters, we can rule this out. Persisters do not form in response to antibiotic treatment, since early exponential cells challenged with antibiotic produce no persisters. Persisters are not cells that temporarily lose their ability to grow due to a reversible defect, such as a stalled replication fork. We would expect defects to occur in early exponential phase as well, though no persisters are formed at this stage. We are left with an interesting possibility of persisters representing specialized survivor cells the production of which is regulated by the growth stage of the population.

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