

# Starvation- and antibiotics-induced formation of persister cells in *Pseudomonas aeruginosa*

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**Background.** Planktonic stationary and exponential cultures of *Pseudomonas aeruginosa* are highly resistant to killing by bactericidal antimicrobials because of the presence of persisters, cells that are multidrug tolerant and play a key role in the recalcitrance of biofilm infections.

**Aim.** The aim of this study was to investigate the formation of persister cells in *P. aeruginosa* stationary vs. exponential cultures using different class antimicrobials.

**Methods.** The susceptibilities of *P. aeruginosa* PAO1 wild-type and mutant strains to antimicrobials were determined by standard microtiter broth dilution method. In order to determine persister formation, dose- and time-dependent killing experiments were performed with antibiotics.

**Results.** Ceftazidime (Cephalosporin) showed little efficacy against either culture. Stationary-phase cells were more tolerant to imipenem (Carbapenem) than exponential cells, leaving a small fraction of persisters at high imipenem concentration in both populations. Polymyxin B (Polymyxin) appeared to be ineffective at low concentrations against both cell populations. Very high polymyxin B concentration completely eradicated exponential cells and regrowth was seen in a stationary population. Stationary cells were more tolerant to tobramycin (Aminoglycoside) than exponential cells but a higher concentration of tobramycin completely eliminated survivors. Ciprofloxacin (Fluoroquinolone) at a low concentration resulted in killing of both cultures of *P. aeruginosa*, producing persisters that were invulnerable to killing.

**Conclusions.** Stationary cells appear to be somewhat more tolerant than exponential cells in all of these assays. We also showed that nutrient deprivation (serine starvation) regulated by stringent and general stress response, contribute to the increased tolerance of *P. aeruginosa* exponential and stationary planktonic cells via production of persisters.

**Key words:** antimicrobials, persisters, tolerance, *P. aeruginosa*, planktonic, serine hydroxamate, mutant

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## INTRODUCTION

*Pseudomonas aeruginosa*, Gram-negative Proteobacteria, is an important nosocomial pathogen and causes a variety of infections, including life-threatening. *P. aeruginosa*, as well as *Escherichia coli* and *Staphylococcus aureus* have been described to produce persisters after treatment with multiple antibiotics<sup>1</sup>.

Persisters are defined as slowly growing or non-dividing cells that are tolerant to killing with lethal doses of bactericidal antibiotics and play an important role in the recalcitrance of chronic infections, when the presence of persisters in biofilms remains undetected by the immune system<sup>2</sup>. In spite of the fact that the persister theory was proposed in the 1940s, much remains to be elucidated about the mechanisms underlying the development of persister formation. However, several global regulators (DnaK, DnaJ, DksA, integration host factor, RelA, SpoT, RpoS) in *E. coli* and *P. aeruginosa* have been demonstrated to influence persisters formation. DnaK and DnaJ are chaperones, DksA is a transcriptional regulator of rRNA transcription and an integration host factor represents a histone-like DNA binding protein which is responsible for the induction of a wide variety of cell processes<sup>3</sup>.

Stationary-phase-related regulators include – (i) RelA and SpoT that control the production of guanosine tetraphosphate, ppGpp, (mediator of bacterial stringent response) and (ii) RpoS (sigma subunit of RNA polymerase). In addition, other genes involved in metabolism, adhesion mediated by type IV pili, DNA replication and repair, virulence, cell death and additional unknown functions have also been implicated in persister formation. These genetic determinants include PhoU (ref.<sup>4</sup>) (repressor of the Pho operon and putative enzyme in phosphate metabolism), alternative sigma factor RpoN, *dinG* (putative DNA helicase), *spuC* (putrescine aminotransferase involved in polyamines metabolism), *algR* (global regulator of cell processes like alginate production; alginate biosynthesis regulatory protein), *pilH* (type IV pilus response regulator), *ycgM* (putative fumarylacetylacetoacetate hydrolase), *pheA* (fused chorismate mutase-prephenate dehydratase involved in phenylalanine biosynthesis and metabolism), PA14\_17880 (putative acetyl-CoA acetyltransferase, PA14\_04150), *dnpA* (de-N-acetylase) and PA14\_13680 (putative short-chain dehydrogenase) (ref.<sup>5-8</sup>). Further cues like biofilm environment, toxins (RelE and MazF) (ref.<sup>2</sup>), quorum-sensing determinants (*lasI* and *lasR*) (ref.<sup>9</sup>) and quorum-sensing-related signaling molecule

acyl-homoserine lactone 3-OC12-HSL and phenazine pyocyanin<sup>10</sup>, were found to be required for persister formation. It is noteworthy that persisters occur in biofilms and planktonic cultures. Seven to twelve type II TA loci have been identified so far in the *P. aeruginosa* chromosome depending on the strain<sup>11</sup> which showed no relevance to persister formation. However, it is possible that more, as-yet-unidentified TA systems exist that are required to enter the persistent state. Regarding stringent response, for *P. aeruginosa* and *E. coli* growing in biofilms it was found that stringent response was necessary for maintaining the low levels of reactive oxygen species (ROS) (ref.<sup>12,13</sup>) which are associated with apoptosis-like death<sup>14</sup>. Furthermore, Aizenman and coworkers described that overexpression of ppGpp induced mazEF death pathway through the stable toxin MazF which is able to exert its deleterious effects and cause cell death<sup>15</sup>. The main advantage of the existence of two cell death pathways appears to be to permit survival of a small part of the bacterial population which may revert to normal growing cells<sup>14,16</sup>.

Persister cells have been reported to be more numerous in stationary than in exponential *P. aeruginosa* cultures isolated by ciprofloxacin<sup>10</sup>. The aim of the present study was to investigate the formation of persister cells in *P. aeruginosa* stationary vs. exponential cultures using different bactericidal antibiotics. Further, we wanted to know (i) how large is the proportion of persisters, (ii) if starvation stress may provoke the persistence phenotype and (iii) to test how general stress response regulator *rpoS* and stringent response genes *relA* and *spoT*, affect persister formation in these cultures. The involvement of stringent response in this phenotype was assessed by starvation-inducing serine analog. Here, we demonstrate that nutrient deprivation (serine starvation) controlled by stringent and general stress response, play a role in antimicrobial tolerance of *P. aeruginosa* exponential and stationary planktonic cells via production of persisters.

## MATERIALS AND METHODS

### Bacterial strains and growth conditions

The bacterial strain used in this study was *P. aeruginosa* PAO1 wild-type,  $\Delta relA$ ,  $\Delta relA spoT$  and  $\Delta rpoS$  strain (kind gift from D. Nguyen<sup>12</sup>). Minimal BM2 succinate medium [62 mM potassium phosphate buffer, pH 7.0, 7 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 50 mM MgSO<sub>4</sub>, 10 mM FeSO<sub>4</sub> and 0.2% (wt/vol) succinate + 0.5% casamino acids] was used to culture PAO1 in all experiments, unless otherwise stated. Succinate was used for the experiments because it is preferable to glucose as carbon source for *Pseudomonas* spp.<sup>17</sup>. LB agar (Difco, Fisher Scientific) was used for culturing. Planktonic stationary-phase cultures were prepared by inoculating with a single colony from a fresh LB agar plate into 5 mL LB broth (Difco, Fisher Scientific) and incubating them overnight at 37 °C with shaking at 220 rpm. Next, 1 mL of portions of overnight culture was centrifuged at 9000 g for 5 min, then washed and resuspended in BM2 medium. Planktonic exponential-phase cultures were prepared by inoculating 10<sup>5</sup> cells/mL and

were grown to an optical density at 600 nm (OD<sub>600</sub>) of 0.6 to 0.8 in BM2 medium.

### Antibiotics

The bacterial cells were exposed to the following antimicrobials: polymyxin B sulfate (Sigma), tobramycin sulfate salt (Sigma), ciprofloxacin (Fluka), ceftazidime and imipenem at the concentrations listed throughout the article.

### Susceptibility testing

The minimal inhibitory concentration, MIC, of each antibiotic was determined by the broth microdilution method according to Wiegand<sup>18</sup>.

### Persistence assay

Persisters were determined by exposure of stationary or mid-exponential cultures (prepared as described above) to antibiotics. Following the challenge, the samples were washed with BM2 medium and diluted in the same medium. Further, twelve and half microliter samples were serially diluted in BM2 and plated on LB agar plates at time zero to obtain the starting colony forming units (CFU/mL). Antibiotics were added at concentrations of five-, ten-, eighty-times the MIC, and killing was assessed at specific time points (1, 3, 5 and 24 h) by colony counting and calculating percent survival relative to untreated cells at time zero. Killing curves for each antibiotic were performed to identify the antibiotic concentrations enabling survival of a small drug-tolerant subpopulation.

### Determination of persister fractions under different condition

To test the effect of serine hydroxamate (SHX; Sigma-Aldrich) on *P. aeruginosa* persister formation, we cultivated *P. aeruginosa* to early logarithmic-phase (2 h 30 min to 2 h 45 min; OD<sub>600</sub> of 0.2–0.3) and then added 500  $\mu$ M SHX to the sample to induce amino acid starvation. After further cultivation to mid-exponential growth phase (an additional 1 h), the proportion of persister cells was determined as mentioned above.

### Statistics

The statistical significance was determined using a Student t test and a *P* value of <0.05. Each experiment was performed in triplicate.

## RESULTS

We chose to probe the presence of persisters in both stationary and exponential *P. aeruginosa* PAO1 cultures with several different bactericidal antibiotics specifically directed against *P. aeruginosa* such as – ciprofloxacin, a fluoroquinolone; ceftazidime, a cephalosporin; imipenem, a carbapenem; tobramycin, an aminoglycoside; and polymyxin B, a polymyxin. The MIC values for PAO1 wild-type are listed in Table 1. In some cases, treatment with increasing concentrations of several antibiotics for 24 h showed typical biphasic killing curves, that revealed

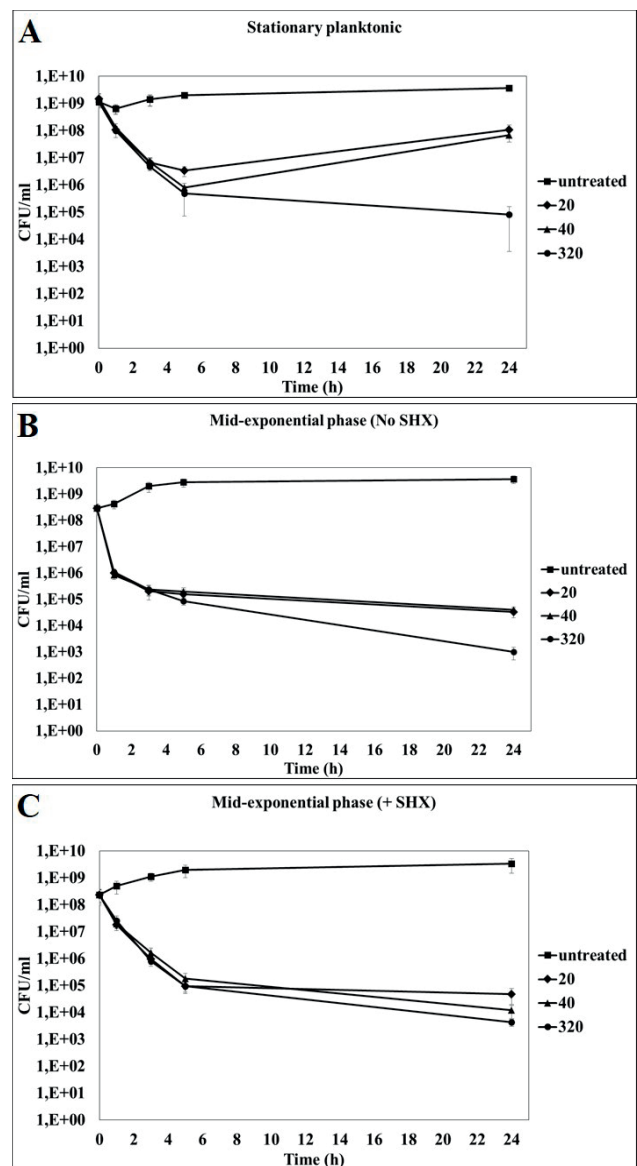
**Table 1.** Antibiotic resistance profile of wild-type *P. aeruginosa* PAO1 strain. MIC ( $\mu\text{g/mL}$ ) for each antibiotic is shown.

Antibiotic	Wild-type
Tobramycin	1.3
Ciprofloxacin	0.6
Ceftazidime	1.1
Polymyxin B	0.8
Imipenem	4

rapid killing of the majority of the cultures, leaving a small persister subpopulation unaffected.

Imipenem, another  $\beta$ -lactam antibiotic, was used to examine the relative tolerance to stationary- and exponential-phase wild-type cultures. Cultures were challenged with imipenem over a wide range of concentrations, from the five- (20  $\mu\text{g/mL}$ ) to eighty-times the MIC (320  $\mu\text{g/mL}$ ). After 1, 3, 5 and 24 h incubation with the antibiotic, bacterial viability was determined by CFU counting. The majority of exponential-phase cells were killed ( $P < 0.0345$ ) at five times the MIC, followed by survival curve that reached a plateau (where the persister fraction did not change when the concentration was further increased) of surviving persisters of this  $\beta$ -lactam antibiotic (Figure 1B). Imipenem in stationary cultures showed an initial rapid bacterial killing of *P. aeruginosa* but regrowth occurred after 5 h at five- and ten-times MIC of imipenem, indicating that these cells formed a larger number of antibiotic-tolerant cells. However, imipenem at very high concentration (320  $\mu\text{g/mL}$ ) produced significant killing ( $P < 0.0001$ ) of stationary culture (Fig. 1A). Taken together, stationary-phase cells were more tolerant to the imipenem than exponential cells (Fig. 1A/B).

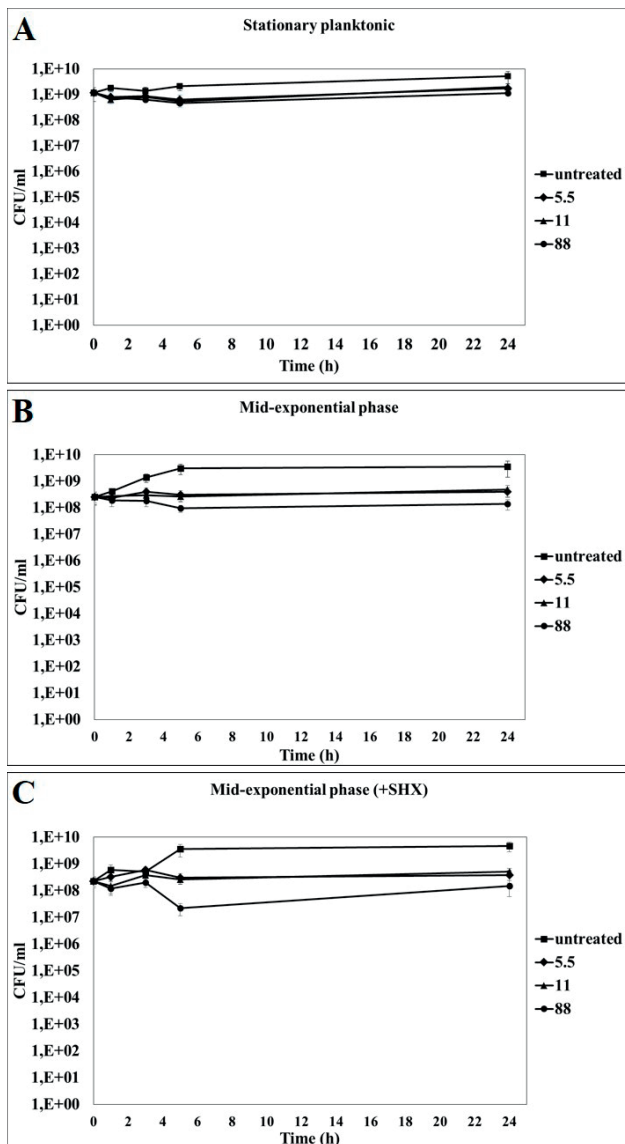
Because of the small number of persisters, we speculated that certain environmental conditions might increase the proportion of persister cells. *P. aeruginosa* PAO1 persister formation was investigated under starvation stress condition. Since SHX influences the levels of stringent response signaling molecule ppGpp in *P. aeruginosa*, we decided to examine its effect on persister formation. This signal molecule is synthesized by RelA and SpoT proteins<sup>19</sup>. Again, SHX (500  $\mu\text{M}$ ) treated cells were treated with identical imipenem concentrations as in Fig. 1B. The imipenem killing curve of exponential *P. aeruginosa* cultures grown with SHX (Fig. 1C) was similar to that without SHX. Only  $\sim 0.007\%$  and  $\sim 0.00035\%$  of drug-tolerant persisters survived 320  $\mu\text{g/mL}$  imipenem treatment for 24 h in stationary- and exponential-cultures (untreated with SHX), respectively, while the exponential cultures of SHX treated *P. aeruginosa* PAO1 increased the fraction of persisters ( $\sim 0.0018\%$ ) (data not shown). Addition of 500  $\mu\text{M}$  SHX to exponential-phase cells resulted in *P. aeruginosa* cultures with a  $\sim 5$ -fold higher proportion of cells surviving imipenem killing. However, this phenomenon (higher proportion of persisters in exponential cells after the addition of SHX to that without SHX) was not observed in cultures exposed to 40  $\mu\text{g/mL}$  (ten-times the MIC) (Fig. 1B/C). These findings support the notion that SHX positively affects the formation of persister cells

**Fig. 1.** Killing of *P. aeruginosa* PAO1 wild-type cells by imipenem.

Cells of stationary-phase (A), exponential-phase untreated with SHX (B) and exponential-phase cultures treated with SHX (C) were exposed to 20-, 40- and 320- $\mu\text{g/mL}$  of imipenem (MIC is 4  $\mu\text{g/mL}$ ) for 1, 3, 5 and 24 h and then plated for colony forming units (CFU/mL). The percent survival is not shown. The values are averages of three independent experiments, and the error bars indicate the standard error of the mean.

in *P. aeruginosa* cultures treated with very high doses of antibiotic (Fig. 1C).

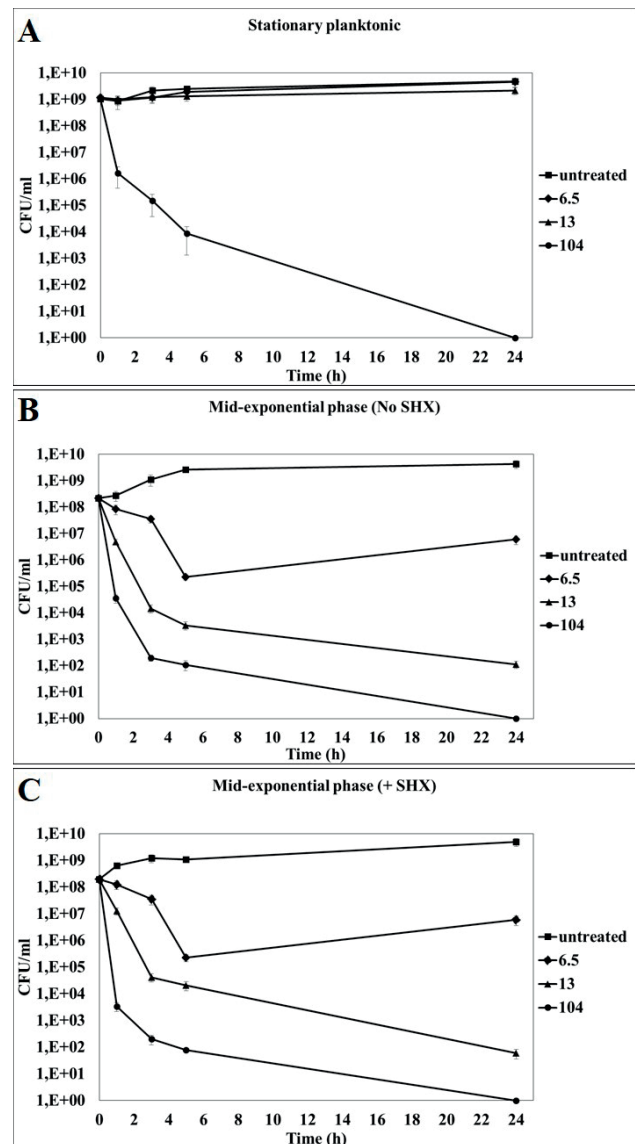
Ceftazidime, a cell wall synthesis inhibitor, is a bactericidal antibiotic that, like other  $\beta$ -lactams, kills rapidly growing cells. If stationary-phase cells are slow growing, they should be resistant to killing by ceftazidime. Stationary and exponential-phase wild-type cells were challenged with 5.5-, 10-, 88- $\mu\text{g/mL}$  concentrations of ceftazidime, which was five-, ten-, and eighty-times the MIC, respectively. Note that MIC experiments treat  $5 \times 10^5$  cells whereas kill curves treat  $2.6 \times 10^8$  and  $1.2 \times 10^9$  cells in exponential and stationary growth phase,



**Fig. 2.** Killing of *P. aeruginosa* PAO1 wild-type cells by ceftazidime.

Cells of stationary-phase (A) and exponential-phase untreated with SHX (B) were exposed to 5.5-, 11- and 88- $\mu\text{g/mL}$  of ceftazidime (MIC is 1.1  $\mu\text{g/mL}$ ) for 1, 3, 5 and 24 h and then plated for colony forming units (CFU/mL). The percent survival is not shown. The values are averages of three independent experiments, and the error bars indicate the standard error of the mean.

respectively. After 1, 3, 5 and 24 h incubation with the antibiotic, killing was assessed by counting colonies. As expected, ceftazidime produced little killing in stationary-phase cells. However, little or no killing was also seen in the exponential phase cultures (untreated and pretreated with SHX) (Fig. 2). Moreover, cultures were tolerant to very high ceftazidime concentration (eighty times MIC) although the presence of resistant cells was evident. These cells were not persisters because no typical biphasic killing with fraction of persisters was observed. We found ceftazidime to be ineffective in killing mid-exponential- and stationary-phase planktonic cells.

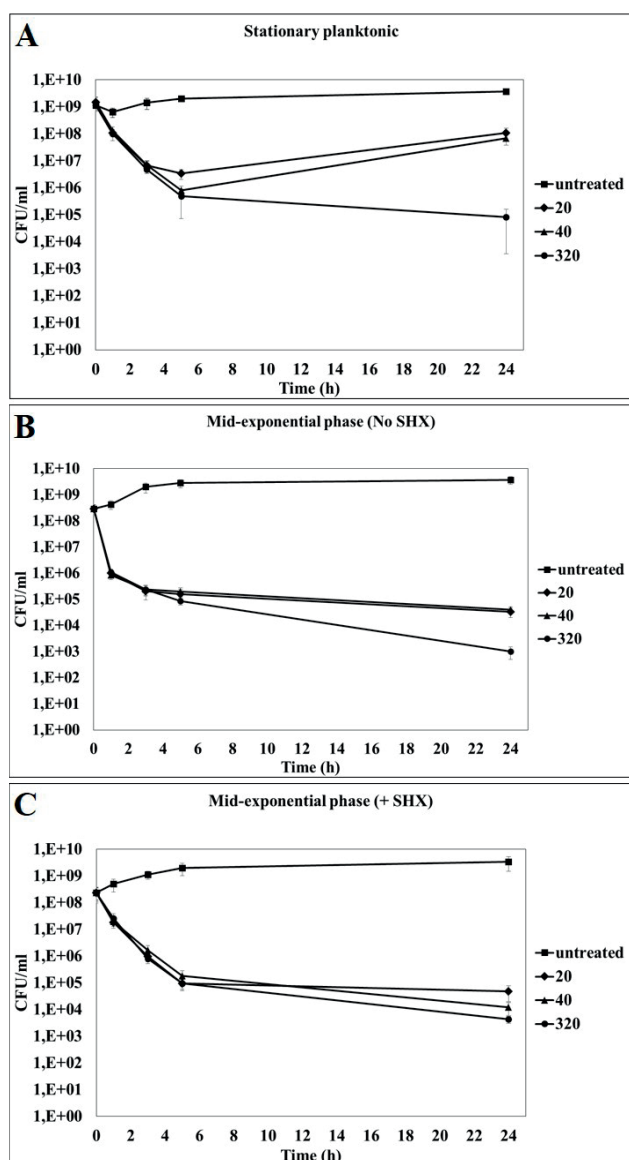


**Fig. 3.** Killing of *P. aeruginosa* PAO1 wild-type cells by tobramycin.

Cells of stationary-phase (A), exponential-phase without SHX (B) and exponential-phase cultures with SHX (C) were exposed to 6.5-, 13- and 104- $\mu\text{g/mL}$  of tobramycin (MIC is 1.3  $\mu\text{g/mL}$ ) for 1, 3, 5 and 24 h and then plated for colony forming units (CFU/mL). The percent survival is not shown. The values are averages of three independent experiments and error bars are not shown, and the error bars indicate the standard error of the mean.

Unlike  $\beta$ -lactams, tobramycin is a bactericidal antibiotic which is able to kill nongrowing cells. Thus, we expected stationary-phase planktonic would be sensitive to killing by this antibiotic. The mechanism of action of tobramycin includes binding with the 30S ribosomal subunit of prokaryotic ribosomes, interrupting translation (creation of toxic misfolded peptides), followed by altered cell membrane permeability and cell death. It also induces ROS production and cell death. All three populations were challenged with tobramycin over a range of concentrations, from the five- (6.5  $\mu\text{g/mL}$ ) to eighty-times the MIC (104  $\mu\text{g/mL}$ ). After 1, 3, 5 and 24 h incubation with

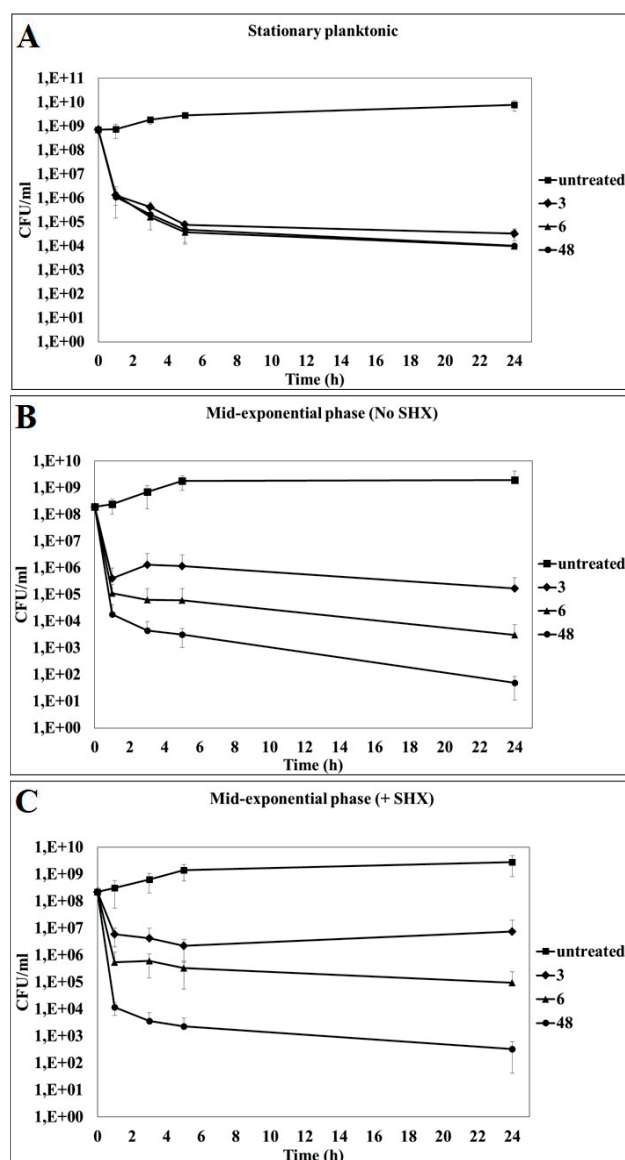




**Fig. 4.** Killing of *P. aeruginosa* PAO1 wild-type cells by polymyxin B.

Cells of stationary-phase (A), exponential-phase without SHX (B) and exponential-phase cultures with SHX (C) were exposed to 4-, 8- and 64- $\mu\text{g/mL}$  of polymyxin B (MIC is 0.8  $\mu\text{g/mL}$ ) for 1, 3, 5 and 24 h and then plated for colony forming units (CFU/mL). The percent survival is not shown. The values are averages of three independent experiments and error bars are not shown, and the error bars indicate the standard error of the mean.

the antibiotic, cell numbers were determined by colony counting. Unexpectedly, tobramycin was not effective in killing stationary-phase cells at concentrations between 6.5 and 13  $\mu\text{g/mL}$  (Fig. 3A). Unlike the stationary-phase cells, the regrowth of the exponential cells were seen after 5 h at five times MIC (6.5  $\mu\text{g/mL}$ ), whereas the majority of these cells ( $P < 0.0056$ ) were eliminated at 13  $\mu\text{g/mL}$  (Fig. 3B). The regrowth indicates the development of acquired resistance to this antibiotic. However, it was very effective in killing all three populations (no persisters) at the highest concentration (104  $\mu\text{g/mL}$ ) (Fig. 3A/B). The killing dynamics of SHX pretreated cultures were similar to those observed without SHX (Fig. 3C).



**Fig. 5.** Killing of *P. aeruginosa* PAO1 wild-type cells by ciprofloxacin.

Cells of stationary-phase (A), exponential-phase without SHX (B) and exponential-phase cultures with SHX (C) were exposed to 3-, 6- and 48- $\mu\text{g/mL}$  of ciprofloxacin (MIC is 0.6  $\mu\text{g/mL}$ ) for 1, 3, 5 and 24 h and then plated for colony forming units (CFU/mL). The percent survival is not shown. The values are averages of three independent experiments and error bars are not shown, and the error bars indicate the standard error of the mean.

Polymyxin B is another bactericidal antibiotic that kills bacteria through membrane lysis and is effective against nongrowing cells. All three populations of wild-type *P. aeruginosa* PAO1 were challenged with polymyxin B over a wide range of concentrations, from the five- (4  $\mu\text{g/mL}$ ) to eighty-times the MIC (64  $\mu\text{g/mL}$ ). After 1, 3, 5 and 24 h incubation with the antibiotic, viability was determined by colony counting. Polymyxin B appeared to be ineffective at low concentrations (five- and ten-times the MIC) against all cell populations (produced little or no killing in the three cultures) (Fig. 4). Polymyxin B was more effective in killing at very high concentration (64  $\mu\text{g/mL}$ , eighty times the MIC) which completely eradicated

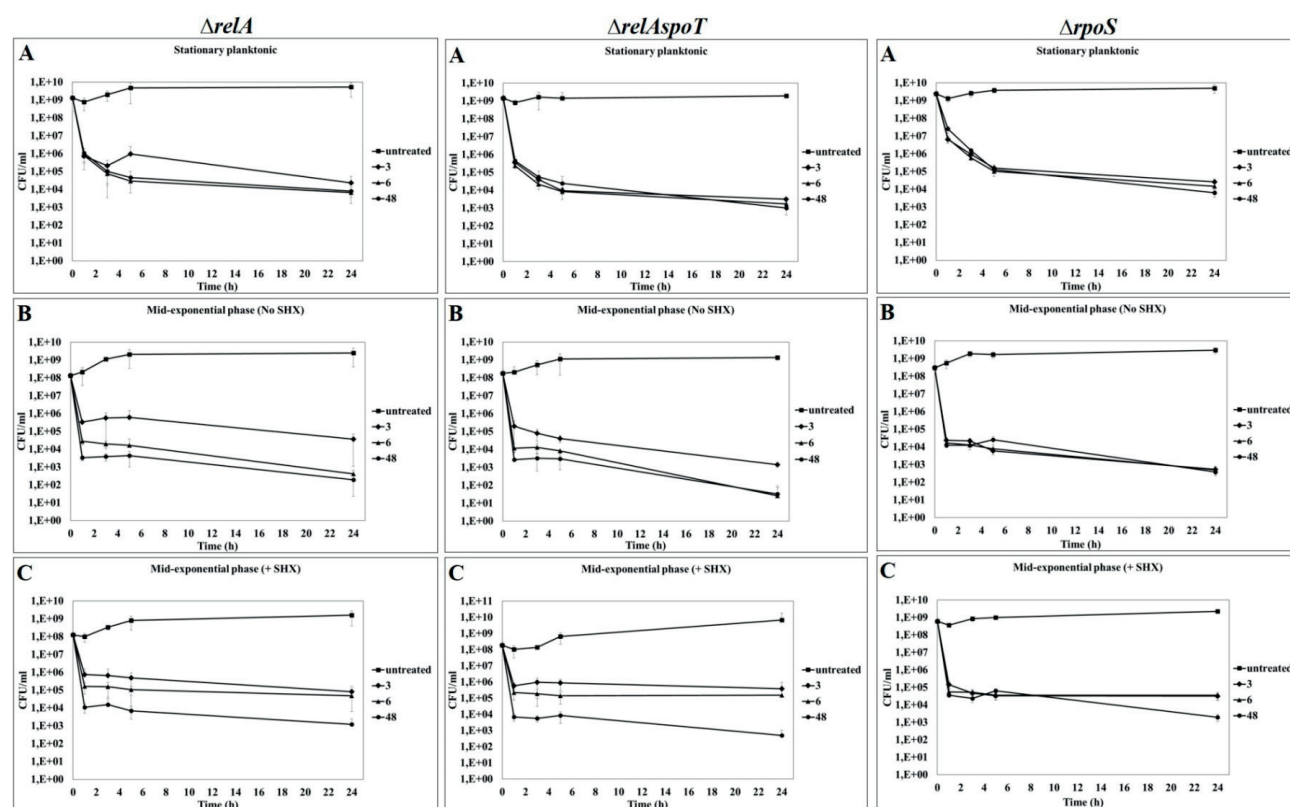
the cells in mid-exponential cultures pretreated and untreated with SHX (no persisters resistant to killing were detected, Fig. 4B/C), whereas the regrowth of stationary *P. aeruginosa* cells was observed after 1 h duration of the experiment (Fig. 4A).

Ciprofloxacin is another bactericidal antibiotic that acts by interfering with DNA replication and can kill non-growing cells. Stationary-phase and exponential-phase wild-type cultures (treated and untreated with SHX) were challenged with ciprofloxacin over a wide range of concentrations, from the five- (3 µg/mL) to eighty-times the MIC (48 µg/mL). After 1, 3, 5 and 24 h incubation with the antibiotic, viability was determined by colony counting. The majority of cells in stationary-phase population examined were killed ( $P < 0.0211$ ) by a concentration of 3 µg/mL ciprofloxacin (Fig. 5A) while ciprofloxacin at higher concentration (6 µg/mL) produced killing of exponential-phase planktonic cells (Fig. 5B/C). Treatment with high concentrations of antibiotic, ten- and eighty-times the MIC, resulted in biphasic killing curves, showing rapid killing of normal cells followed by a plateau of surviving persisters (Fig. 5). These 0.0014%, 0.0016% and 0.043%, of the wild-type stationary culture, exponential culture untreated and pretreated with SHX, respectively, were invulnerable to killing by ciprofloxacin at 6 µg/mL (data not shown). In comparison, only 0.0014%, 0.000025% and 0.00015% of drug-tolerant persisters cells survived 48 µg/mL ciprofloxacin treatment in stationary- and exponential *P. aeruginosa*

**Table 2.** Antibiotic resistance profile of *P. aeruginosa* PAO1 *relA*, *relAspoT* and *rpoS* mutants. MIC (µg/mL) for each antibiotic is shown.

Antibiotic	<i>relA</i> mutant	<i>relAspoT</i> mutant	<i>rpoS</i> mutant
Ciprofloxacin	0.6	0.6	0.6

cultures untreated and pretreated with SHX, respectively (data not shown). This effect was dose-dependent, reaching 6- to ~27-fold increase of persister cells in exponential cultures pretreated with SHX over untreated cells when 80- and 10-times the MIC of ciprofloxacin was used, respectively (data not shown). As in the case of imipenem, cultivation of mid-exponential cultures of *P. aeruginosa* PAO1 in the presence of SHX affected the persister fraction (Fig. 5C). This elevation of persister numbers in *P. aeruginosa* cultures is probably not a consequence of growth arrest because ciprofloxacin is active against non-growing cells, and/or decreased susceptibility to ciprofloxacin but the activation of stringent response. The general effect of SHX on growth of *P. aeruginosa* cultures has been described elsewhere<sup>12</sup>. Shortly, addition of 500 µM SHX to exponential cultures of wild-type *P. aeruginosa* increased the number of antibiotic-tolerant bacteria by over ~2300-fold in ofloxacin treated cells. Moreover, general stress response regulator, *rpoS* gene, is associated with persistence in *P. aeruginosa*<sup>6</sup>.



**Fig. 6.** Killing of *P. aeruginosa* PAO1 *relA*, *relAspoT* and *rpoS* mutants by ciprofloxacin.

Cultures were exposed to 6 µg/mL of ciprofloxacin for 1, 3, 5 and 24 h and then plated for colony forming units (CFU/mL). The percent survival is not shown. The values are averages of three independent experiments and error bars are not shown, and the error bars indicate the standard error of the mean.

The observations that SHX can increase the fraction of persisters in ciprofloxacin cultures suggested a direct role of stringent response in regulating persisters numbers. To test whether the loss of RpoS and (p)ppGpp production would alter the persister proportion, different knockout strains -  $\Delta rpoS$ ,  $\Delta relA$  and  $\Delta relAspoT$  double-deletion mutant were used in this study (all knockout strains, except the first one, were reported to exhibit a lack of stringent response<sup>12</sup>). Interestingly, ppGpp stimulates accumulation of RpoS in *P. aeruginosa*<sup>20</sup>. The MIC values for PAO1 *relA*, *relAspoT* and *rpoS* mutants are listed in Table 2. The persister levels in mid-exponential- and stationary-cultures of the PAO1 strain and different knockout strains were then examined. Mid-exponential cells (pretreated and untreated with SHX) and stationary cultures were challenged with ciprofloxacin over a wide range of concentrations, from the five- (3  $\mu\text{g/mL}$ ) to eighty-times the MIC (48  $\mu\text{g/mL}$ ). After 1, 3, 5 and 24 h incubation with the antibiotic, viability was determined by colony counting. Interestingly, wild-type,  $\Delta rpoS$  and all the deletion strains with decreased or completely eliminated (p)ppGpp production were able to survive challenge with high doses of an antibiotic (Fig. 5 and 6). Biphasic killing indicates the presence of drug-tolerant persisters in all the cultures examined. These findings suggest that even though the stationary-phase sigma factor RpoS and stringent response genes are able to modulate persister formation in *P. aeruginosa*, additional mechanisms exist for mediating persistence.

We next compared the killing of all *P. aeruginosa* PAO1 wild-type and mutant stationary and mid-exponential phase cells (pretreated and untreated with SHX) by the antibiotic ciprofloxacin at 6  $\mu\text{g/mL}$  (ten times MIC). We found that the proportion of persisters was mostly the highest in the mid-exponential phase cells pretreated with SHX, followed by stationary- and exponential-phase cells untreated with SHX in all tested strains except for the wild-type strain when the percentage of persisters was the highest in the exponential culture pretreated with SHX followed by exponential culture untreated with SHX and stationary population (data not shown). Note that pre-treatment of exponential-phase cells with SHX appeared to produce more persisters than the stationary planktonic cells. On the other hand, the proportion of persisters was the highest in the wild-type strain, followed by  $\Delta relA$ ,  $\Delta rpoS$  and  $\Delta relAspoT$ , as shown by comparison of levels of CFU after challenge with 6  $\mu\text{g/mL}$  of ciprofloxacin in all *P. aeruginosa* mid-exponential phase cells untreated with SHX (data not shown), while no differences were detected in MIC (Table 2). These 0.0015%, 0.0003%, 0.00017% and 0.00002% of the wild-type,  $\Delta relA$ ,  $\Delta rpoS$  and  $\Delta relAspoT$  exponential cultures, respectively, were invulnerable to killing by ciprofloxacin at 6  $\mu\text{g/mL}$  (data not shown). Interestingly, a *P. aeruginosa* deletion double mutant lacking the *relA* and *spoT* genes ( $\Delta relAspoT$ ) produced numbers of persisters in all growth stages and also exhibited the SHX-mediated increase in exponential-culture persisters, suggesting that RelA and SpoT do not play a major role in the formation of persisters in PAO1.

## DISCUSSION

*P. aeruginosa* is highly resistant to antibiotic treatment which is achieved through the combination of different factors (such as chromosomally-encoded  $\beta$ -lactamase, extended-spectrum  $\beta$ -lactamase and aminoglycoside-modifying enzymes). Further, the *P. aeruginosa* is able to grow in planktonic and/or biofilm mode of growth. The latter is advantageous for pathogenicity/colonization and resistance to antibiotic treatment. Other mechanisms such as the development of swarming colonies, the stringent response, i.e. response to nutrient limitation, and the formation of multiresistant subpopulation of persistent cells contribute to the multidrug-resistant phenotype<sup>21-23</sup>. Furthermore, *P. aeruginosa* is versatile in the development or acquisition of new mechanisms of resistance to antibiotic treatment. These facts suggest that *P. aeruginosa* has a wide arsenal that enables survival in environments with antibiotics.

Spoering and Lewis<sup>24</sup> demonstrated that stationary-phase planktonic cells of *P. aeruginosa* were somewhat more tolerant than biofilms toward antibiotic killing and suggested that tolerance to antimicrobials is determined by the presence of persister cells. Persisters are not usual resistant mutants and yet they survive antibiotic treatment. After antibiotic treatment, persister cells can revert to normally growing cells and cause relapse of infections. Although, several genes were found to be involved in persister formation, the additional regulatory factors remain to be elucidated. Is the proportion of persisters really higher in stationary or exponential cells? We therefore performed a comparison of the persister numbers in mid-exponential and stationary phase cells of *P. aeruginosa* PAO1 by treatment with up to eighty times the MIC of ceftazidime, imipenem, polymyxin B, tobramycin and ciprofloxacin for 24 h.

In our experiments, imipenem, a  $\beta$ -lactam antibiotic, at low concentrations produced significant killing ( $P < 0.0345$ ) of exponential-phase planktonic cells. However, stationary-phase cells were more resistant to killing than were exponential cultures and regrowth was observed five hours after the initial rapid reduction in viable cells at concentrations up to 40  $\mu\text{g/mL}$  (Fig. 1A). Imipenem produced biphasic killing in all three cultures (in stationary culture at very high concentration), indicating the presence of persister cells resistant to killing (Fig. 1). In exponential-phase cells untreated with SHX and challenged with 320  $\mu\text{g/mL}$  imipenem for 24 h, the portion of *P. aeruginosa* survivor cells ( $\sim 0.00035\%$ ) was 5-fold lower than that of mid-exponential-phase cells pretreated with SHX ( $\sim 0.0018\%$ ) and 20-fold lower than that of stationary-phase cultures ( $\sim 0.007\%$ ) (data not shown). This finding indicates that serine starvation contributes to persister formation. This is in agreement with a report by Nguyen<sup>12</sup>, who found that serine starvation increased the number of viable persister cells after challenge with ofloxacin, a fluoroquinolone antibiotic that binds topoisomerases and causes DNA damage. In this context, it is interesting to note that carbapenems are strong inducers of *ampC* and genes encoding the RND efflux pumps (MexXY-OprM,



as well as MexAB-OprM or MexCD-OprJ), and those with other mechanisms (e.g., loss of porin protein D) may contribute to carbapenem resistance<sup>25</sup>. Moreover, the *rpoS* mutant strain has been associated with increased susceptibility to carbapenems in stationary phase cells of *P. aeruginosa*<sup>6</sup>. This implies the involvement of RpoS in carbapenem resistance. Last but not least, RpoS has been linked to persisters formation in *P. aeruginosa*<sup>5</sup>.

Ceftazidime, another  $\beta$ -lactam antibiotic, was ineffective in killing both the stationary- and exponential-phase planktonic cells (Fig. 2). Slow growth, production of chromosomal AmpC  $\beta$ -lactamase and/or efflux pumps might explain resistance to killing by this antibiotic<sup>26</sup>. However, bacterial survival may be also affected by very high bacterial load.

Tobramycin is capable of killing nongrowing cells. However, it is known to be ineffective in killing biofilm cells because of its binding to biofilm exopolysaccharide<sup>27,28</sup>. In fact, increase in growth of *P. aeruginosa* exponential phase cells was noted at 5 hours for the 6.5  $\mu$ g/mL of the antibiotic tobramycin (Fig. 3B/C). At very high tobramycin concentrations (eighty-times the MIC), the bacterial cells were completely killed after 24 h and no persisters were seen in any tested culture, where the 13  $\mu$ g/mL trend in exponential cultures appeared as though it would kill the cells as well (Fig. 3). Our results showed that a stationary culture is more resistant to killing than are exponential-phase cells. Although this stationary-phase cell population was washed and resuspended in fresh medium, exopolysaccharide was probably still present in the culture. Interestingly, Allison and coworkers<sup>29</sup> reported that persisters cells in planktonic and biofilm cultures of *E. coli* and *Staphylococcus aureus* can be sensitized to aminoglycosides by the addition of specific carbon sources (e.g., glucose, mannitol) which recover proton-motive force for aminoglycoside uptake.

Polymyxin B had no effect on stationary- or exponential-phase cells in the presence of antibiotic levels five- and ten-fold higher than the MIC. Interestingly, polymyxin B at very high concentration (eighty-times the MIC) in stationary culture showed an initial rapid killing but regrowth occurred after 5 h, whereas no persisters were seen (all bacteria were eradicated) in the exponential-phase cells untreated and pretreated with SHX (Fig. 4). Interestingly, *cbrA* mutant has been shown to be associated with enhanced resistance towards polymyxin B, colistin, ciprofloxacin and tobramycin<sup>17</sup>. Colistin is now used as the last option in the treatment of microorganisms with multiple resistance. The effect of antibiotic lies in the disruption of the cytoplasmic membrane. However, resistance to this antibiotic has already been described and is based on changes in the lipopolysaccharide in the outer membrane due to mutations in regulatory genes, e.g. *phoQ* gene<sup>30</sup>.

Ciprofloxacin at concentration ten-times the MIC produced killing of all tested cultures of wild-type PAO1 but a small percentage of persisters cells resistant to killing was evident (Fig. 5). We found that ciprofloxacin at ten-times the MIC exhibited biphasic killing and the numbers of persisters were  $\sim$ 28-fold higher in exponential culture pretreated with SHX than in the untreated cells, compris-

ing 0.043% and 0.00156% of the population, respectively (data not shown). However, when concentrations of ciprofloxacin up to 80-times the MIC were used, the portion of *P. aeruginosa* survivor cells ( $\sim$ 0.000025%) in exponential-phase cells untreated with SHX was 6-fold lower than pretreated with SHX and about 56-fold lower than that of stationary-phase cultures ( $\sim$ 0.00139%) (data not shown). It is noteworthy that in these experiments stationary culture was transferred into a fresh medium, essentially without dilution due to a higher number of persisters, as described elsewhere<sup>24</sup>. Unexpectedly, exponential-phase planktonic cells pretreated with SHX and challenged with ciprofloxacin at 6  $\mu$ g/mL produced relatively more persisters than did a stationary culture and were more tolerant to ciprofloxacin. These findings suggest that the persister formation may be associated with starvation conditions as well as density of the population, probably in response to quorum-sensing factor(s). Regarding the above results, ceftazidime, a cephalosporin antibiotic, showed reduced efficacy in treating slow-growing cells and no persisters were seen. Polymyxin and tobramycin completely killed the cells, whereby the cells were invulnerable to killing by imipenem and ciprofloxacin. This highlights the importance of protein synthesis before chromosome replication. It has been described elsewhere that (i) the presence of antibiotics (e.g. fluoroquinolone) induces persister formation by inducing SOS response<sup>31</sup> and toxin-antitoxin genes<sup>32</sup>. However, other antibiotics like  $\beta$ -lactams and aminoglycosides have been also shown to induce the SOS response<sup>33</sup>. Further, it was reported that killing of *E. coli* by  $\beta$ -lactams and quinolones followed similar changes in the transcription profile<sup>34</sup> and it is assumed that there are common genes which are associated with antibiotic activity and are controlled by ppGpp and RpoS. Interestingly, Maisonneuve and his colleagues reported that deletion of five or more toxin-antitoxin systems increased bacterial susceptibility to  $\beta$ -lactams and fluoroquinolones<sup>35</sup>. Point mutations in the *gyrA* gene confer resistance to quinolones<sup>36</sup>. In addition, subinhibitory concentrations of ciprofloxacin have been found associated with the development of adaptive resistance<sup>37</sup>.

Serine starvation activates the stringent response by the overexpression of *relA* and *spoT* in *P. aeruginosa*, leading to elevated production of (p)ppGpp. Nguyen and coworkers demonstrated that stringent response mediates the suppression of the oxidative stress by both restricting the production of pro-oxidants and inducing antioxidant defenses, leading to the persistence in *P. aeruginosa*<sup>12</sup>. Further studies on ciprofloxacin-induced persister formation were performed under all three conditions using the stringent response- and stationary-phase sigma factor mutants;  $\Delta relA$ ,  $\Delta relAspoT$  and  $\Delta rpoS$ . A  $\Delta relAspoT$  double deletion mutant lacks the ability to produce (p)ppGpp. Vice versa, (p)ppGpp accumulation was present only in the wild-type strain<sup>12</sup>. It is well known that RpoS is involved in a number of processes, including energy metabolism, motility, stress responses and antibiotic tolerance through regulation of SOS response and mutagenesis<sup>38</sup>. Moreover, association between stringent response and RpoS has been described when ppGpp stimulates accumulation of RpoS in *P. ae-*



*ruginosa* during the stationary phase<sup>20</sup>. We found that all these strains were able to survive the antibiotic treatment up to eighty-fold the MIC and induced persister formation. In  $\Delta relA$  and  $\Delta relAspoT$  mutants, serine starvation reduced killing by only  $\sim 114$ - and  $5500$ -fold after challenge with ciprofloxacin at ten-times the MIC, respectively (data not shown). Whereas stationary-phase growth of wild-type,  $\Delta relA$  and  $\Delta relAspoT$  *P. aeruginosa* produced  $\sim 10^4$ ,  $<10^4$  and  $\sim 10^3$  ciprofloxacin-tolerant bacteria, respectively (Fig. 5A and 6A). Our results further showed that  $\Delta rpoS$  cultures pretreated with SHX and challenged with ciprofloxacin at ten-times the MIC displayed increased persistence ( $\sim 35$ -fold increase over cultures not treated with SHX) (data not shown). However, the proportion of surviving cells was the highest in exponential-phase planktonic cells pretreated with SHX, followed by stationary culture and exponential-phase cells untreated with SHX in all tested strains, except for the wild-type strain, challenged with ciprofloxacin at ten-fold and eighty-fold the MIC (data not shown). On the other hand, numbers of persisters were the highest in the wild-type strain (0.00156%),  $\Delta relA$  ( $\sim 0.0003\%$ ),  $\Delta rpoS$  ( $\sim 0.00017\%$ ) and  $\Delta relAspoT$  (0.000015%), when mid-exponential phase cells untreated with SHX were exposed to ciprofloxacin at ten-fold the MIC (data not shown). It has been described that *rpoS* transcripts were more abundant during in the exponential phase than in the stationary phase of *Legionella pneumophila*<sup>39</sup>. However, high stress or stationary phase upregulates the expression of *relA* and *spoT* genes, whereby ppGpp levels accumulate<sup>40</sup>. These results indicate that the *relA*, *spoT* and *rpoS* genes are important in fluoroquinolone tolerance and are in agreement with results obtained by Nguyen and his colleagues that showed that nutrient-limited and ppGpp-deficient *P. aeruginosa* cultures were less resistant to antimicrobials than their wild-type counterparts<sup>12</sup>. Since the decreased proportion of persisters in double mutant was evident, it can be speculated that not only (p)ppGpp but also other signaling molecule(s) may regulate and enhance persister formation to protect *P. aeruginosa* population from complete killing by antibiotics, whereby SHX may stimulate these molecule(s). The direct role of SHX in persistence of ppGpp-deficient mutant of *P. aeruginosa* remains to be elucidated. The effect of starvation stress in environments, as well as in biofilms, appears to be important in persister formation. Further, SOS and SOS-induced toxin-antitoxin system may be the main mechanism controlling persister formation through DNA damage responsible for genetic recombination and mutations<sup>31,41</sup>. Much remains to be elucidated about the mechanisms underlying the development of cell death and persister formation in *P. aeruginosa*. Last but not least, these and other studies show that not only biofilm cells but also stationary planktonic cells may be used for identification of genes involved in persisters formation.

## CONCLUSION

A comparative study of adaptive resistance in *P. aeruginosa* stationary and exponential planktonic cells in the presence of the antibacterial drugs revealed that two

bactericidal antibiotics – imipenem and ciprofloxacin, induce persister formation. Other antibiotics, polymyxin B, ceftazidime and tobramycin, appeared to be less effective at low concentrations against growing and non-growing cells but the higher concentration of antibiotic was more effective against all tested cultures (tobramycin) and exponential cells (polymyxin B; regrowth in stationary population). In general, stationary culture was more tolerant than exponential-phase cells. Our data further demonstrate that starvation-inducing serine analog, SHX, increased the persister numbers in exponential PAO1 cultures of all tested strains challenged with imipenem (at very high concentration) and ciprofloxacin against cultures untreated with SHX and resulted in increased tolerance to these antibiotics. The other important, unexpected, conclusion of this study is that mutant cells of *P. aeruginosa* in exponential growth phase pretreated with SHX before the treatment with ciprofloxacin are more tolerant to antibiotics than the stationary planktonic cells. Interestingly, *RpoS* mutant and (p)ppGpp-defective cultures were able to survive ciprofloxacin treatment and induce persisters. Taken together, this phenotype can be affected by stringent response, nutrient deprivation (serine starvation) and general stress response. The formation of persisters indicates that additional mechanism(s) are involved in the regulation of persister numbers in stationary and exponential cultures. Better understanding of cell death pathways, as well as genes involved in persister formation, may pave the way for the development of new antimicrobial agents and improve the treatment of chronic *P. aeruginosa* infections.

## ABBREVIATIONS

ROS, reactive oxygen species; SHX, serine hydroxamate; MIC, minimal inhibitory concentration; ppGpp, guanosine tetraphosphate.

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