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Assessing *Pseudomonas aeruginosa* Persister/Antibiotic Tolerant Cells

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Abstract

Bacterial persistence, which is observed in a broad range of microbial species, is the capacity of a bacterial cell subpopulation called “persisters” to tolerate exposure to normally lethal concentrations of bactericidal antibiotics. This ability, which is not due to antibiotic-resistant mutants, has been implicated in antibiotic treatment failures and may account for latent, chronic, and relapsing infections. Antibiotic tolerant/Persister (AT/P) cells have been notoriously difficult to study due to their low frequency and transient nature. This chapter describes the main methods used to isolate and study *Pseudomonas aeruginosa* AT/P cells and discusses new technologies that may ease research of *P. aeruginosa* persisters in the near future.

Keywords

Antibiotic tolerance; Bacterial persistence; Quorum sensing; Small molecules; High-throughput screen; Antibiotics; Anti-virulence; Anti-infectives; Proliferation; Virulence; Infection

1 Introduction

Bacterial persistence is the capacity of bacterial cell subpopulations to tolerate exposure to normally lethal stresses, including lethal concentrations of bactericidal antibiotics [1]. These cells represent a small subpopulation of cells that behave as dormant, latent, or viable but nonculturable (VBNC) bacterial cells and have been observed in a variety of microbial species since the 1940s [2–4]. Of particular clinical interest is multidrug tolerance, which is a phenomenon that has been implicated in treatment failures and accounts for latent, chronic, and relapsing infections that can be suppressed but not eradicated [5]. Unlike multidrug resistance, antibiotic tolerance is not due to mutations, but rather to phenotypic variations. Persisters can later resume growth when the antimicrobial agent is removed, and their progeny remains sensitive to low concentrations of antimicrobial agents [1, 6–8]. The clinical importance of antibiotic tolerance is reflected by the many cases in which antibiotics failed to clear infections despite the absence of resistant bacteria, and clinical reports suggest that the contribution of tolerance to treatment failure and mortality in some infections can be as significant as the contribution of antibiotic resistance.

Pseudomonas aeruginosa is one of the most well-studied bacteria, as it is a recalcitrant pathogen known to defy bactericidal eradication and exemplifies clinically problematic pathogens that cause both acute and chronic human infections [9]. This pathogen does not exhibit the canonic toxin–antitoxin systems involved in *E. coli* persisters [9, 10]. However, several other pathways, such as the metabolism of polyamines, fatty acids, phospholipids,

phenylalanine, and DNA as well as global regulators, translation, quorum sensing, and antioxidant responses have been identified as having roles in the formation of *P. aeruginosa* persisters [11–23].

In this chapter, we describe the main techniques and necessary steps for isolating and quantifying *P. aeruginosa* persister cells.

2 Methods

2.1 Selection and Quantification of Antibiotic Tolerant Persister Cells

Here, we describe the protocols used to evaluate the size of the persister fraction within a bacterial culture.

1. Grow starter cells from a –80 °C stock in 5 mL LB at 37 °C overnight (*see* Note 1).
2. Dilute the cells 1:100 in 5 mL tubes and grow them under the desired conditions to a defined optical density.
3. Determine the concentration of viable bacteria within the culture [expressed as colony forming unit (CFU)/mL] according to Subheading 2.2 before antibiotic addition. This number will be used as a normalization reference ("normalizers") to calculate the percentage of cells that survived antibiotic treatment (surviving fraction).
4. Add antibiotic at a concentration of 10–100 times its specific minimal inhibitory concentration (MIC) as determined in Subheading 2.2. (*see* Note 2).
5. Incubate the cells in a shaker (200 rpm) at 37 °C for 24–48 h (*see* Fig. 1).
6. Transfer 500 μL of each sample to 1.5 μL Eppendorf tubes and centrifuge for 500 μL of each sample for 5 min at 8,000 × *g*.
7. Remove supernatant gently without touching the pellet. Sometimes the pellet is invisible due to very low persister concentrations.
8. Wash by adding 1 mL of phosphate-buffered saline (PBS), centrifuge for 5 min at 14,000 ×*g*, and gently discard the supernatant.
9. Depending on antibiotic concentration, repeat **step 8** two to four times in order to ensure that the antibiotic has been washed out (*see* Note 3).
10. Resuspend the pellet in 500 μL of an appropriate medium.
11. Determine the surviving fraction (antibiotic tolerant persister cells) according to Subheading 2.1.1.
12. Validate that the surviving bacteria are indeed tolerant cells and not resistant mutants as described in Subheading 2.3.

2.1.1 Determination of Bacterial Number and Surviving Fractions—The gold standard method to determine bacterial concentration in a sample is the CFU plating

method, which consists of plating several dilutions for each sample on LB agar plates [24]. However, this method is very time consuming and tedious in the case of high-throughput screens, for which the drop plate and *s*tart of *g*rowth *t*ime (SGT) methods are more appropriate ("Drop Plate Method" and "SGT Method") [25, 26].

CFU Plating Method (*See* Note 4)

1. Perform eight 1:10 serial dilutions of a bacterial sample in Eppendorf tubes. The number of dilutions can be reduced in cases where the number of bacteria can be estimated.
2. Plate triplicates of 100 μL of each dilution on agar plates containing the appropriate medium.
3. Incubate plates for 24–48 h at 37 °C.
4. Count the colonies on each plate and calculate the CFU/mL as follows: number of colonies × 10 × $10^{-\text{dilution}}$.

Drop Plate Method (*See* Note 5)

1. Fill three columns of a 96-well plate per sample with 270 μL of LB, except the first row of wells.
2. Add 200 μL of sample in the three empty wells of the first row of wells.
3. Remove 30 μL from the wells of the first row using a multichannel pipette and place into 270 μL of LB in the wells of the second row (dilution 1:10).
4. Perform serial dilutions of the sample by repeating **step 3** down to the bottom row seven times using a multichannel pipette. This gives a range of dilutions from non-diluted (upper row) to a 10^{-7} dilution (bottom row). Use a second 96-well plate if needed to dilute samples to a factor greater than 10^{-7}.
5. Using a multichannel pipette, place drops of 10 μL on an agar plate. One plate can contain up to 6 rows of 7 dilutions. The agar plate surface should be dry enough to avoid blending of the dilution drops.
6. Let the plate dry before incubation at 37 °C for 24 h.
7. Count the colonies and calculate CFU/mL as follows: number of colonies × 100 × $10^{-\text{dilution}}$.

SGT Method (*See* Note 6)

1. For both untreated normalizer and persister cell quantification, add 2 μL of each sample into 1 mL of LB in Eppendorf tubes (1:500 dilution).
2. Transfer 200 μL from each tube into three wells (technical triplicate) of a 96-well plate.
3. Incubate the plate in a plate reader set to measure the optical density (OD) at a 600 nm wavelength every 15 min for 24 h at 37 °C with a 10 s shaking step every 15 min.

4. Define the SGT value of each sample as the time required for the culture to reach an $OD_{600\ nm}$ threshold that is set slightly above the detectable background at the start of the logarithmic phase of growth (usually OD_{600nm} = 0.15 or 0.2).

5. Calculate the first ΔSGT value for each sample according to the following equation: $\Delta SGT = (SGT_{persisters} - SGT_{Normalizers})$, where the SGT of untreated normalizer cells is subtracted from the SGT of treated persister cells.

6. Calculate the ΔΔSGT value by subtracting the ΔSGT of the reference strain/condition ("calibrator") from that of the sample as follows: $\Delta\Delta SGT = (\Delta SGT_{Sample} - \Delta SGT_{Calibrator}$

7. Calculate the fold change between the sample and the calibrator as the following: $F = 2^{-\Delta\Delta^{SGT}}$. Results are presented as $\log_2$ fold changes: –ΔΔSGT (*see* Fig. 2).

2.2 Determination of the Antibiotic Concentration to Be Used for Selection of Persisters

1. In order to select the persister subpopulation, the culture needs to be exposed to bactericidal levels of the antibiotic. MIC of each antibiotic could be determined according to the standard protocol [27]. However, in the case of persisters it is important to determine the killing concentration of the antibiotic in the conditions and strains of the experiment. Here we present a simple method to estimate the selective concentration of the antibiotics in specific conditions. Grow cells from a –80 °C stock in the desired conditions overnight for 18 h (usually LB at 37 °C with shaking at 200 rpm).

2. Dilute the cells 1:1,000 in the same media the next day.

3. Inoculate 100 μL of cultures in 3 rows of transparent 96-well plates that will serve as technical triplicates using a multichannel pipette (e.g., rows A–C).

4. Prepare antibiotic stock. A good starting range for the concentration of the antibiotic would be between 10 and 100 mg/mL.

5. Dilute the antibiotic stock 1:500 to 20–200 μg/mL with the media containing bacteria from **step 2**.

6. Add 100 μL of the diluted antibiotic to wells A1, B1, and C1 and mix thoroughly by pipetting up and down.

7. Replace the pipette tips and transfer 100 μL from wells A1–C1 to wells A2–C2.

8. Repeat **step 7** for all wells to A11–C11. Remove the excess 100 μL from the last row and discard.

9. Retain column 12 without antibiotic as a positive control. The plate now contains a 2,048 × range of concentrations between the first well and well 11.

10. Incubate the plate for 24 h at 37 °C and read its $OD_{600\ nm}$ using a plate reader.

11. The specific MIC would be the lowest concentration of antibiotic that did not permit growth of bacteria.

2.3 Confirm that the Surviving Bacteria Are Indeed Tolerant and Not Resistant Mutants

After the first round of bacteria selection by temporary exposure to the antibiotic, the surviving colonies need to be retested to assess that they are indeed tolerant and not resistant mutants. This is done by checking the MIC and by measuring the size of the persister fraction of cultures started from surviving cells again as described in Subheadings 2.1 and 2.2.

3 Additional Methods

The major disadvantage of the culture-based persisters assessment techniques is that they indirectly determine the number of dormant cells based on the number of surviving cells by measuring the growing progeny. More direct methods to analyze dormant cells include microscopy [28], flow cytometry [21, 29], and microfluidics [19, 30] together with the use of probes that allow for the distinction between live and dead cells, even if the cells are not dividing [31]. In order to study persister "wake-up," a method using automated scans termed "ScanLag" has been described for *E. coli,* which could also be applied to *P. aeruginosa* [32].

4 Notes

1. The incubation time for the starter cultures from a –80 °C stock should always be the same duration, because bacterial cultures can accumulate persisters as incubation progresses [1]. This important precaution will help avoid having inconsistent results between experiments.
2. As a rule of thumb, the concentration of antibiotic used to select for tolerant cells is at least 10 times the MIC. It should be noted that the MIC method determines the resistance of cells to the specific antibiotic, rather than their tolerance to antibiotic-induced killing. The distinction between these two effects is whether the cells are growing in the presence of antibiotic or only surviving the antibiotic killing. Table 1 shows the MICs of commonly used antibiotics against *P. aeruginosa* with the strain PA14 grown in LB at 37 °C.
3. In order to determine the size of the antibiotic-tolerant cell fraction, the antibiotic needs to be completely removed from the culture. If the expected concentration of the fraction of bacteria surviving is greater than 1,000 cells/mL, then removal of the antibiotic used at ~100× MIC can be carried out by making ~100-fold serial dilutions. However, if the concentration of the fraction of bacteria surviving is ≤100–1,000 cells/mL, then dilutions of less than ~100-fold would result in a carryover of antibiotic, thereby restricting bacterial growth on agar plates, and >100-fold dilutions would result in bacterial concentrations that are too low for detection. Therefore, a cautious step of cell wash out needs to performed in order to remove the antibiotic without reducing the amount of detectable bacteria in samples, as described in steps **6–10** in Subheading 2.1.
4. The advantages of this CFU plating method are that only viable bacteria are counted and the dilutions allow for any number of bacteria to be counted regardless of the starting concentration. One limitation of the CFU method is that

clumps of bacterial cells can be miscounted as single colonies. In addition, CFU is usually not the method of choice for a high-throughput screen because it is a relatively time-consuming and tedious.

5. Using the drop plate method described here, the bacteria are first diluted in 96-well plates and 10 μL drops are then plated. Thus, instead of using 42 plates, an array of 6 × 7 drops can be easily spotted on a single standard Petri dish. One disadvantage of this method is the reduction in accuracy, but it can be overcome by plating multiple technical replicates.

6. The SGT method is a quantitative method [33]. It combines the methodology of quantitative polymerase chain reaction (qPCR) calculations [34, 35] with a previously described qualitative method of bacterial growth determination [12] to develop an improved quantitative method [33]. The SGT method allows rapid and serial quantification of the absolute or relative number of live cells in a bacterial culture in a high-throughput manner. The SGT method is based on the regrowth time required by a growing cell culture to reach a threshold (spectrophotometrically detectable levels), and the notion that this time is proportional to the number of cells in the starting bacterial inoculum [12, 33] (*see* Fig. 2).

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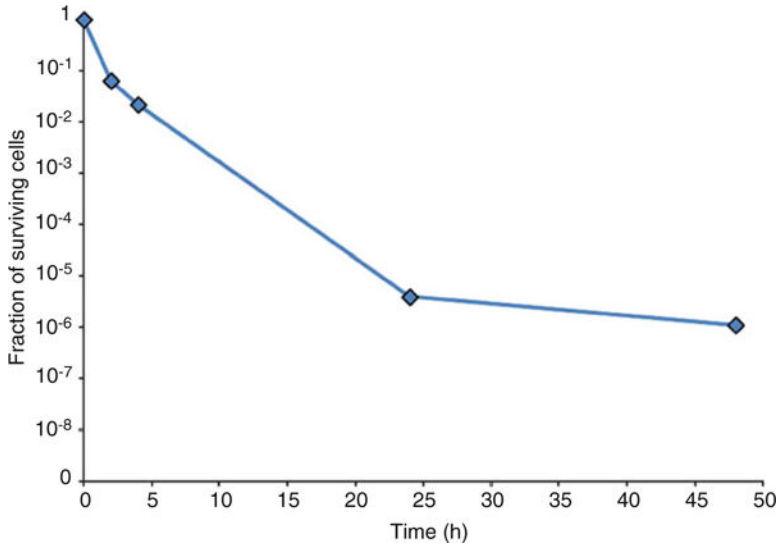

Fig. 1.

Antibiotic tolerant cell assessment using the CFU counts method. The killing curve of *P. aeruginosa* strain PA14 exponential-phase cells exposed to a lethal concentration of the bactericidal antibiotic meropenem indicates that the majority of cells died quickly, showing a sharp drop-off in survival kinetics within 24 h, while a small fraction of cells (~10^{-6}) survived the treatment even after 48 h of antibiotic exposure. This surviving fraction of cells reflects the number of antibiotic tolerant cells. PA14 cells were exposed to 10 mg/L meropenem for 48 h

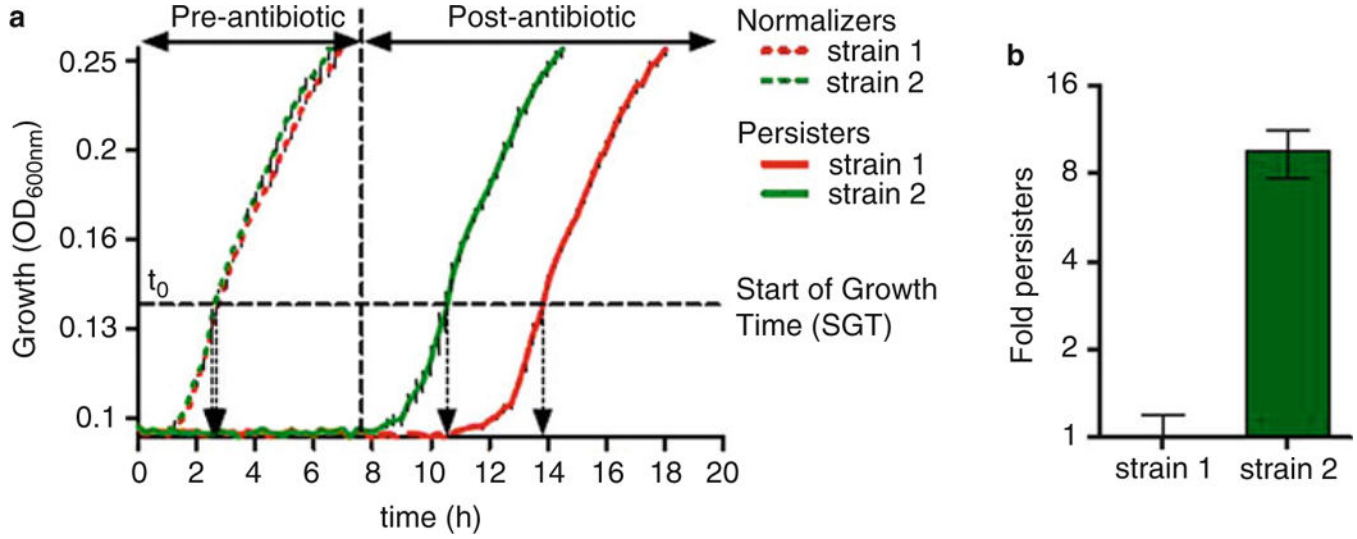

Fig. 2.

Antibiotic tolerant cell assessment using the SGT method. Comparative assessment of the persister cell fraction between two strains subjected to a 24 h treatment with meropenem (10 mg/L) at 37 °C (no meropenem added to normalizers). Following a 1:500 dilution, the growth kinetics of normalizers and treated samples were recorded. (**a**) Using OD_{600nm}=0.15, the ΔSGT values were calculated as the difference between treated and normalizer SGTs. The ΔΔSGT values were calculated as the difference between ΔSGTs of each strain compared to that of the calibrator. (**b**) For the SGT method, the log_2-fold change was calculated as –ΔΔSGT (empty bars). For CFU counting, normalizers and treated cells were serially diluted

Table 1

Minimal inhibitory concentration (MIC) of commonly used antibiotics on PA14

Family	Name	MIC on PA14 μg/mL)
Aminoglycoside	Amikacin	1
	Gentamycin	1.5
Beta-lactam	Imipenem	1
	Meropenem	0.1
Quinolone	Levofloxacin	2
	Ciprofloxacin	0.1