Chapter 2 - Antibiotic tolerance and persistence studied throughout bacterial growth phases

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Summary

Antibiotic tolerance and persistence allow bacteria to survive lethal doses of antibiotic drugs in the absence of genetic resistance. Despite the urgent need to address these phenomena as a cause of clinical antibiotic treatment failure, studies on antibiotic tolerance and persistence are notorious for contradictory and inconsistent findings. Many of these problems are likely caused by differences in the methodology used to study antibiotic tolerance and persistence in the laboratory. Standardized experimental procedures would therefore greatly promote research in this field by facilitating the integrated analysis of results obtained by different research groups. Here, we present a robust and adaptable methodology to study antibiotic tolerance / persistence in broth cultures of *Escherichia coli* and *Pseudomonas aeruginosa*. The hallmark of this methodology is that the formation and disappearance of antibiotic-tolerant cells is recorded throughout all bacterial growth phases from lag after inoculation over exponential growth into early and then late stationary phase. In addition, all relevant experimental conditions are rigorously controlled to obtain highly reproducible results. We anticipate that this methodology will promote research on antibiotic tolerance and persistence by enabling a deeper view at the growth-dependent dynamics of this phenomenon and by contributing to the standardization or at least comparability of experimental procedures used in the field.

1. Introduction

Since their initial observation in the 1940s by Bigger, bacterial antibiotic tolerance and persistence have drawn the attention of researchers and clinicians all over the world ***(1)***. By definition, antibiotic tolerance is the ability of bacteria to survive nominally lethal concentrations of bactericidal antibiotics, while antibiotic persistence denotes the survival of a typically non-growing, antibiotic-tolerant subpopulation among a heterogeneous population of otherwise sensitive, growing cells ***(2)***. Though these phenomena are thought to play major roles in antibiotic treatment failure and the frequent relapses of chronic infections ***(3,4)***, their physiological basis and the underlying molecular mechanisms have remained largely unknown despite extensive research ***(5)***. One major obstacle to progress in this field is that the published literature contains many contradictory or at least inconsistent findings ***(2,5-8)***. Dedicated research has suggested that these issues might be largely due to differences in the experimental methodologies used to assess antibiotic tolerance and persistence in the laboratory ***(2,9-11)***. In particular, the genetic background of the bacterial model organisms, their growth medium and culture conditions, and the inoculation method can have a large impact on the number of cells recovered after antibiotic treatment ***(9-13)***. Recently, the field made an important effort to agree on common definitions of antibiotic tolerance and antibiotic persistence which included fundamental experimental guidelines on how to study these phenomena and how to distinguish them from antibiotic resistance ***(2)***. However, no direct practical advice regarding experimental methodologies was included that could help researchers in the field understand their different results and guide newcomers around common pitfalls.

The methodology presented in this chapter was developed for *Escherichia coli* and *Pseudomonas aeruginosa*, two major model organisms in the field, and largely relies on the rigorous control of all relevant experimental variables by, e.g., using a fully defined growth

medium to avoid comparing "apples, oranges and unknown fruit" ***(14)***. Rather than artificially inducing antibiotic tolerance through some kind of bacteriostatic treatment ***(15-17)***, our methodology solely focuses on antibiotic-tolerant cells that form in response to cues inherent to the bacterial life cycle such as starvation or entry into stationary phase. In addition, our methodology provides a complete view on the dynamics of antibiotic tolerance by recording the formation and disappearance of antibiotic-tolerant cells throughout the bacterial growth phases from lag after inoculation over exponential growth into early and late stationary phase. This comprehensive view enables the comparison of mutant strains with different growth dynamics and can reveal phenotypes that affect tolerance or persistence only in certain growth stages ***(8,18)***. Conversely, the focus on single, defined growth time points for antibiotic tolerance assays is prone to comparing bacteria in different growth stages and blind to whether observed differences between strains are, e.g., due to changes in the formation or in the disappearance of antibiotic-tolerant cells.

2. Materials

Prepare all solutions using autoclaved ultra-pure water (Milli-Q grade) and store all reagents at room temperature in the dark unless indicated otherwise. All solutions, including the antibiotic stock solutions, should be sterilized by filtration through a 0.2 μm filter before use unless indicated otherwise (see **Notes 1 and 2**).

1. 5x M9 salts solution: 33.9 g/L Na_2HPO_4, 15 g/L KH_2PO_4, 5 g/L NH_4Cl, 2.5 g/L NaCl. Dissolve the powder in ca. 80 % of the final volume of the solution, e.g., in 800 mL of water if you are preparing 1 L of solution, in a large beaker. Mix well using a magnetic stirrer. Once all the powder is dissolved, fill up to the desired volume using a graduated cylinder. M9 salts can either be prepared from the individual components

(see **Note 3**) or be bought as premixed powder (Sigma-Aldrich M6030). This solution can be stored for years in the dark.

2. 100x trace elements solution: 0.18 g/L $ZnSO_4 \cdot 7H_2O$, 0.12 g/L $CuCl_2 \cdot 2H_2O$, 0.12 g/L $MnSO_4 \cdot H_2O$, 0.18 g/L $CoCl_2 \cdot 6H_2O$ (equivalent to 0.63 mM $ZnSO_4$, 0.70 mM $CuCl_2$, 0.79 mM $MnSO_4$, and 0.76 mM $CoCl_2$). Weigh the powders into a beaker and dissolve directly into the final volume of water. Store in the dark.
3. 100 mM $FeCl_3$ solution: 16.22 g/L $FeCl_3$. Dissolve the powder directly into the final volume of water and mix well. Store in the dark at 4 °C. $FeCl_3$ is corrosive, so special care should be taken and gloves as well as protective goggles should always be worn when manipulating it.
4. 1 M $MgSO_4$ solution: 246.47 g/L $MgSO_4 \cdot 7H_2O$. Dissolve the powder in the final volume of water and mix well.
5. 1 M $CaCl_2$ solution: 147.01 g/L $CaCl_2 \cdot 2H_2O$. Dissolve the powder in the final volume of water and mix well.
6. 2.2 M or 40 % (w/v) D-glucose solution: 440 g/L D-glucose monohydrate ($C_6H_{12}O_6 \cdot H_2O$). Add the powder to ca. 70 % of the final volume of water. Stir until everything is dissolved, subsequently fill up to the final volume using a measuring cylinder. Store at 4 °C (see **Note 4**).
7. M9Glc or M9-based culture medium (50 mL): 38.75 mL ultra-pure water (sterilized), 10 mL 5x M9 salts solution, 500 µL 40 % w/v D-glucose solution, 500 µL 100x trace elements solution, 30 µL 100 mM $FeCl_3$ solution, 100 µL 1 M $MgSO_4$ solution, 5 µL 1 M $CaCl_2$ solution. Using sterile technique, mix all the substances in the order as listed to avoid precipitation. The final pH should be close to 7.2. Store at 4 °C for up to 1 week. Final molar concentrations of all components are listed in Table 1 (see also **Note 5**). This medium is referred to as "M9Glc medium" throughout the article.

8. PBS (phosphate-buffered saline) solution: 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L $Na_2HPO_4 \cdot 2H_2O$, 0.24 g/L KH_2PO_4. Dissolve in 90 % of the final volume of water and adjust the pH to 7.4 using 10 M NaOH. Fill up with water to the final volume and autoclave to sterilize.
9. LB agar plates: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L agar, square Petri dishes (12 cm x 12 cm), round Petri dishes (9.4 cm diameter). Mix all components and add water to the final volume. Sterilize by autoclaving. After sterilization, let the liquid LB agar cool down to ca. 60 °C and subsequently pour the required number of plates by dispensing 45 mL LB agar per square Petri dish or 24 mL LB agar per round Petri dish. Leave the agar to solidify with the lid closed at room temperature. After one day, the plates are sufficiently dry for usage and can be stored in tightly closed plastic bags at 4 °C for several weeks (see **Notes 6 and 7**).
10. Antibiotic stock solutions (50 mg/mL ampicillin, 1 mg/mL ciprofloxacin, 10 mg/mL tobramycin sulfate, and 5 mg/mL meropenem trihydrate): Dissolve 53.14 mg ampicillin sodium salt, 1 mg ciprofloxacin, 10 mg tobramycin sulfate, and 5.7 mg meropenem trihydrate in 1 mL ultra-pure water each (see **Note 8**). Ampicillin sodium salt, ciprofloxacin and tobramycin sulfate stocks can be prepared in advance and stored at -20 °C for several weeks, but multiple cycles of freezing and thawing should be avoided. Meropenem must be prepared freshly before each use due to low stability of the solution (see **Notes 8-10**).
11. Dilution plates: To prepare for serial dilutions, fill all wells in rows B-H of the required number of columns (one per sample) of a microtiter 96-well plate with 180 µL of PBS. To avoid evaporation, this should be done shortly before the serial dilutions are performed. Keep sterile.

12. Plastic test tubes: At least one sterile 1.5 mL test tube is needed per tested condition and time point. An excess of tubes should be sterilized by autoclaving before the experiment.
13. Flasks and glass tubes: One 50 mL Erlenmayer flask per strain, one 100 mL Erlenmeyer flask per strain, and one cylindrical round-bottom glass culture tube with loose aluminium cap (15 mL nominal volume; 10 x 1.5 cm) per strain and condition are needed to determine levels of antibiotic-tolerant cells along the bacterial growth curve. One 50 mL Erlenmayer flask per strain, one 500 mL Erlenmeyer flask per eight experimental conditions, and one cylindrical round-bottom glass culture tube with loose aluminium cap (15 mL nominal volume; 10 x 1.5 cm) per strain and condition are needed to perform the stationary-phase assay (see **Note 11**).
14. Graduated pipettes: To pipette and transfer larger volumes of media and culture, adequate sterile graduated pipettes should be used.
15. Bacterial strains: The protocols were developed for standard laboratory strains *E. coli* K-12 MG1655 F^- λ^- *ilvG*$^-$ *rfb-50 rph-1* (Coli Genetic Stock Center (CGSC) #6300) and *Pseudomonas aeruginosa* PAO1 *Δpel Δpsl* ***(19)*** (see **Note 12**).
16. Cultivation and incubation: Bacterial liquid cultures should be agitated in a shaking incubator at 37 °C at 170 rpm. Agar plates should be incubated without agitation at 37 C.

Table 1. Composition of M9Glc medium. This table summarizes the components needed to prepare M9Glc medium.

Component	Stock solution	Volume (per 50 mL)	Final concentration
Ultra-pure water	-	38.7 mL	-
5x M9 salts solution	5x	10 mL	1x
D-glucose solution	2.2 M (40 % w/v D-glucose)	500 µL	2.2 mM (0.4 % w/v D-glucose)
Trace elements solution	100x	500 µL	1x
$FeCl_3$ solution	100 mM	30 µL	0.06 mM
$MgSO_4$ solution	1 M	100 µL	2 mM
$CaCl_2$ solution	1 M	5 µL	0.1 mM

3. Methods

In this part, the experimental procedures to determine antibiotic tolerance of *E. coli* K-12 MG1655 and *P. aeruginosa* PAO1 are described. In the first section, we describe the procedure to determine levels of antibiotic-tolerant cells of a bacterial culture followed from lag to early stationary phase. In the second section, we describe the procedure to determine levels of antibiotic-tolerant cells in deep stationary phase.

3.1. Determination of antibiotic tolerance along the growth curve

3.1.1. Day 0 – Collection of materials and inoculation of the overnight culture

1. Prepare or collect all the materials needed for the assay (step 1 in section 3.1.3 and following) (Table 2).

2. Pick an isolated bacterial colony and use it to inoculate 5 mL of M9Glc medium in a 50 mL Erlenmeyer flask. Incubate the pre-culture for 24 h at 37 °C shaking (170 rpm) (see **Notes 11-13**).

Table 2. Materials per strain tested. The table summarises the materials and media needed per strain for the experiment "Determination of antibiotic tolerance along the growth curve" (section 3.1).

M9Glc medium	ca. 100 mL
phosphate-buffered saline (PBS) solution	ca. 70 mL
Glass culture tube	8
LB-agar square Petri dish	2
100 mL Erlenmeyer flask	8
1.5 mL plastic test tube	16
96-well microtiter plate	2
5 mL graduated pipette	16

3.1.2. Day 1 – Subculturing

1. Dispense 10 mL of M9Glc medium into one 100 mL flask and let the medium reach room temperature.
2. Dilute back the pre-culture 1:100 into the flask prepared in the previous step. Incubate this secondary pre-culture at 37 °C shaking (170 rpm) for 24 h (see **Notes 11 and 12**).

3.1.3. Day 2 – Antibiotic tolerance assay

1. Switch on the shaking incubator (37 °C / 170 rpm) at least 30 min before the start of the experiment to let it heat up. In the meantime, take the M9Glc medium out of the fridge, aliquot it into the glass flasks using sterile technique, and let the medium reach

room temperature. While waiting for the medium to warm up, the tubes needed for the assay can be labelled and other preparatory work can be done.

2. Dilute the pre-culture 1:100 into 10 mL of fresh M9Glc medium in a 100 mL Erlenmeyer flask and culture both the subculture and the pre-culture at 37 °C / 170 rpm. Repeat this subculturing step after 2 h and then six more times in a way that, eventually, eight cultures inoculated 8 h, 6 h, 5 h, 4 h, 3 h, 2 h, and 1 h before the last one are available (see **Notes 11, 12 and 14**).
3. Determine the concentration of colony forming units (CFU/mL) in the eight cultures first directly to assess the total number of bacteria (section 3.1.3 steps 4-6) and, subsequently, after antibiotic treatment (section 3.1.3 steps 7-10) to assess the number of survivors (section 3.1.4 and following).
4. Transfer 100 µL of each culture into the wells of row A of a 96-well microtiter plate previously filled with 180 µL PBS. Perform 1:10 serial dilutions with the multichannel pipette by transferring 20 µL of the cultures from one row to the next containing PBS. Mix well and change tips after every dilution (Fig. 1, left).
5. Use a multichannel pipet to transfer 10 µL spots of all dilutions from the 96-well plate onto a dry LB agar square Petri dish (see **Note 6**; Figure 1, right)
6. Wait for the spots to dry with open lid (e.g., next to a Bunsen burner or under a flow hood). Once the spots have dried, incubate the plate at 37 °C for 16-24 h before counting.
7. For the antibiotic treatment, transfer 3 mL of each subculture from the flask into a glass tube and challenge with antibiotics at the desired final concentration. We typically treat with 100 µg/mL ampicillin (*E. coli*) or 12 µg/mL meropenem (*P. aeruginosa*), 10 µg/mL ciprofloxacin, and 40 µg/mL tobramycin sulfate. Place the eight tubes in the incubator (37 °C / 170 rpm) for 5 h (see **Notes 11, 15 and 16**).

8. After 5 h of antibiotic challenge, transfer 1.5 mL of each culture to a sterile 1.5 mL plastic tube and pellet the cells by centrifugation for 2 min at 18.000 g. Remove the supernatant carefully and wash each pellet with 1 mL of sterile PBS. Start by washing the samples with the lowest incubation times and pay attention to the small, barely visible pellets that result from low-density cultures (see **Notes 17 and 18**). Repeat the centrifugation, remove the supernatant, and finally resuspend the pellets in 100 µL of sterile PBS.
9. Dilute and spot these samples obtained from the antibiotic-treated samples as described in section 3.1.3 steps 4 and 5 (see Figure 1).
10. Let the spots dry and incubate the plate at 37 °C for at least 16 h but monitor colony formation for 48 h (see Figure 2 and **Notes 19 and 20**).

3.1.4. Day 3 – CFU counting

1. After 16-24 h of incubation, select the spots that contain between 10 to 100 bacterial colonies. Count the CFU per spot (CFU/spot). Subsequently, put the plates back for an additional incubation of ca. 24 h at 37 °C (see Figure 2 and **Note 19**).

3.1.5. Day 4 – CFU counting and plotting

1. Check the plates from day 3 and, if necessary, update the CFU counts. Subsequently, calculate the CFU/mL values for all samples before and after antibiotic treatment.
2. Optional: Calculate the fraction of antibiotic-tolerant cells at each time point as the ratio of CFU/mL after and before antibiotic treatment.
3. To obtain independent biological replicates, the experiment should be performed at least three times on different days.
4. When sufficient biological replicates have been performed, the data can be averaged and the standard error of the mean can be calculated. To calculate the average,

transform all the CFU/mL values into their base 10 logarithm. Consequently, calculate the standard error of the mean on the individual log-transformed values.

5. Plot the recovered CFU/mL for each time point before and after antibiotic treatment on a base 10 logarithmic scale against time of growth before treatment (Fig. 3). If enough replicates have been performed, plot the mean values instead and include the respective error values as well. Note that these curves *per se* only report on antibiotic tolerance and that the inference of antibiotic persistence requires additional time kill curves (see **Note 20**). The results and their interpretation are briefly discussed in **Note 21**.

3.2. Determination of antibiotic tolerance in stationary phase

3.2.1. Day 0 – Preparation of media and cultures

1. Prepare or collect all the materials needed for the assay (section 3.2.3 step 2 and following) (Table 3).
2. Pick an isolated bacterial colony and use it to inoculate 5 mL of M9Glc medium in a 50 mL Erlenmeyer flask. Incubate the pre-culture for 24 h at 37 °C shaking (170 rpm) (see **Notes 11-13**).

Table 3. Materials needed for the "Determination of antibiotic tolerance in stationary phase" experiment. The table summarises the materials and media needed per strain for the experiment "Determination of antibiotic tolerance in stationary phase" (section 3.2).

M9Glc medium	ca. 60 mL
Phosphate-buffered saline (PBS) solution	ca. 50 mL
Glass culture tube	8
LB-agar square Petri dish	6
100 mL Erlenmeyer flask	2
500 mL Erlenmeyer flask	2
1.5 mL plastic test tube	40
96-well microtiter plate	6
5 mL graduated pipette	2

3.2.2. Day 1 – Subculturing

1. Dispense 50 mL of M9Glc medium into one 500 mL Erlenmeyer flask and let the medium warm up to room temperature.
2. Dilute back the pre-culture 1:100 into the flask prepared in the previous step. Incubate the subculture at 37 °C shaking (170 rpm) for 48 h (see **Notes 11 and 12**).

3.2.3. Day 3 – Stationary-phase antibiotic tolerance assay

1. For the antibiotic treatment, transfer 5 mL of each culture from the Erlenmeyer flask into a glass tube and challenge with antibiotics at the desired final concentration. We typically treat with 100 µg/mL ampicillin (*E. coli*) or 12 µg/mL meropenem (*P. aeruginosa*), 10 µg/mL ciprofloxacin, and 40 µg/mL tobramycin sulfate. Place the tubes in a shaking incubator (37 °C / 170 rpm) (see **Notes 11, 15 and 16**). Always include one untreated sample as a control for bacterial stationary-phase viability.
2. To determine viable cell counts, 200 µL aliquots are withdrawn from each glass tube at different time points, typically after 0 h (i.e., before addition of the antibiotics), 1 h, 3 h, 7 h, 24 h, as well as 48 h, and transferred into a 1.5 mL tube. Each tube is centrifuged for two min at 18.000 g and washed once with 200 µL of sterile PBS to remove excess antibiotic.
3. Transfer 100 µL of each culture into the wells of row A of a 96-well microtiter plate previously filled with 180 µL PBS. Perform 1:10 serial dilutions with the multichannel pipette by transferring 20 µL of the cultures from one row to the next containing sterile PBS. Mix well and change tips after every dilution (similar as shown in Fig. 1, left).
4. Use a multichannel pipet to transfer 10 µL spots of all dilutions from the 96-well plate onto a dry LB agar square Petri dish (see **Note 6**; Figure 1, right).

5. Wait for the spots to dry with open lid (e.g., next to a Bunsen burner or under a flow hood). Once the spots have dried, incubate the plate at 37 °C for 16-24 h.

3.2.4. Day 4 – CFU counting

1. After 16-24 h of incubation, select the spots that contain between 10 to 100 bacterial colonies. Count the CFU per spot (CFU/spot). Subsequently put the plates back for an additional incubation of ca. 24 h at 37 °C (see Figure 2 and **Note 19**).

3.2.5. Day 5 – CFU counting and plotting

1. Check the plates from day 4 and, if necessary, update the CFU counts. Subsequently, calculate the CFU/mL values for all samples before and after antibiotic treatment.
2. To obtain independent biological replicates, the experiment should be performed at least three times on different days.
3. Plot the recovered CFU/mL for each time point on a base 10 logarithmic scale against time of treatment (Fig. 4). If enough replicates have been performed, plot the mean values instead and include the respective error values as well. The results and their interpretation are briefly discussed in **Note 22**.

4. Notes

1. Sterilization by filtration should always be preferred since autoclaving can alter the chemical composition of solutions. This is particularly important for solutions containing complex molecules such as glucose which can degrade with heat ***(20)***.
2. All experimental steps, from the preparation of the media to the antibiotic killing assays, should be performed according to the rules of good laboratory practice ***(21)***.

3. If preparing the M9 salts solution from the individual components, be aware of water of hydration inside the salt crystals (e.g., disodium hydrogen phosphate is usually available as $Na_2HPO_4 \cdot 7H_2O$) and adapt the recipe accordingly to preserve molarities. The recipe described in this article corresponds to the quantities used by Sigma-Aldrich to prepare their variant of M9 salts powder (Sigma-Aldrich M6030). Note that there are some minor differences between M9 salts recipes that are circulating in the community, e.g., between the recipe of Sigma-Aldrich and the one formulated by Cold Spring Harbor Protocols ***(22)***.
4. Dissolving the glucose may take up to 1 h. It is not recommendable to add water to dissolve the glucose powder more quickly, as the volume of the solution increases considerably when the D-glucose is dissolving. Instead, heating the solution gradually up to 40 °C can help dissolving the powder more quickly.
5. The main benefit of using a chemically defined medium prepared with ultra-pure water is that the same cellular physiology can be obtained in every experiment and among different laboratories. This is often not the case for complex media that may suffer from batch-to-batch variation due to poorly defined ingredients (e.g., tryptone and yeast extract) or due to degradation of some components if stored or prepared improperly ***(8,12)***. The trace element supplement is adapted from previous work by Gerosa et al ***(23)***. Unless strictly necessary, we do not suggest to supplement the medium with thiamine (as it is done in many M9 medium recipes including the one of Gerosa et al ***(23)***) in order to avoid that this compound might be used as an alternative carbon or nitrogen source. To assess antibiotic tolerance of thiamine auxotrophs with the present protocol it is enough to supplement the medium with thiamine to a final concentration of 2.8 μM (as described in reference ***(23)***). Any other auxotrophies can be complemented analogously.

6. Drying the plates at room temperature for up to 48 h (not wrapped in a plastic bag) generates a more hygroscopic surface which avoids that drops of bacterial samples spotted closely flow into each other and merge. Furthermore, a dry agar plate will cause bacterial samples to soak into the agar more quickly.
7. Previous experience of the authors showed that *P. aeruginosa* is very sensitive to the brand of agar used to prepare LB agar plates. Depending on the brand and the overall concentration of agar used, the colony morphology of *P. aeruginosa* and surface-dependent behaviours like swarming motility can be very different. In this work, AppliChem A0949 agar was used. If it is not possible to use this brand, it might be worth to test a few different brands or concentrations (13 g/L – 17 g/L) to see which one gives an optimal colony size after 24 h of incubation at 37 °C (3–5 mm). Notably, *E. coli* seems to be not visibly affected by agar brand and concentration.
8. Antibiotic powders are often provided in form of a salt (e.g., ampicillin sodium salt). In these cases, antibiotic stocks should be prepared so that the final concentration of the stock corresponds to the actual antibiotic concentration and not to the concentration of its salt. When preparing ciprofloxacin antibiotic stock from pure ciprofloxacin powder, a few drops of 1 M HCl must be added to help complete dissolution of the powder in ultra-pure water. Alternatively, it is also possible to purchase ciprofloxacin hydrochloride monohydrate which is readily soluble in water.
9. *P. aeruginosa* is intrinsically resistant to ampicillin due to an inducible AmpC β-lactamase ***(24)***. Therefore, a different β-lactam antibiotic must be used to assess the tolerance of *P. aeruginosa* to β-lactams. We propose to use meropenem, but also other β-lactams can be used such as imipenem, piperacillin, or cefepime.
10. Antibiotic tolerance and persistence can either be confined to single drugs or affect multiple antibiotics, and the levels of antibiotic-tolerant cells can vary widely among

different strains of the same species ***(25-27)***. In order to obtain a complete picture, we therefore recommend to always separately assess tolerance to antibiotics with different modes of action such as β-lactams, fluoroquinolones, and aminoglycosides that are commonly used in the field (see also Fig. 3 and 4).

11. The proper aeration of a culture during long incubation times is essential to avoid uncontrolled changes of bacterial physiology that could affect outcome of the antibiotic tolerance assay. Therefore, make sure to cultivate the bacteria in a volume of medium that is around 10 times less than the nominal fill volume of the Erlenmeyer flasks. Shaking tubes and flasks at 170 rpm is suitable to achieve both proper aeration and to prevent sedimentation of the bacteria. Whenever having a culture in a non-self-sustaining container (e.g., a glass culture tube), this should be inclined at ca. 45° to ensure optimal aeration. The inclination should be kept constant for the whole duration of the assay.
12. *P. aeruginosa* can form small aggregates in liquid batch culture that exhibit biofilm-like characteristics including, e.g., increased antibiotic tolerance and therefore distort the results of antibiotic treatment assays. However, the formation of these aggregates can be largely avoided by using a mutant deficient in production of Pel and Psl exopolysaccharides as well as by using pre-cultures rather than direct inoculation from a single colony or cryostocks ***(9)***. Nevertheless, visible aggregates of *P. aeruginosa* can transiently form during the pre-cultures and subcultures but disperse again upon starvation ***(28)***. Therefore, take care while pipetting to not include visible bacterial aggregates in the inocula for subcultures.
13. The colonies should be inoculated from a freshly streaked plate that is not older than one week. Alternatively, the pre-culture can also be inoculated directly from a

cryostock. It is advisable to keep the inoculation method consistent among experiments, as it may have an influence on the results ***(9,11)***.

14. It is recommended to prepare, collect, and label wherever necessary all the materials that are required throughout the steps prior to experimentation.
15. Measure the antibiotic minimum inhibitory concentration (MIC) by broth dilution assays in 96-well microtiter plates of the tested strain to make sure that the concentration used for the antibiotic tolerance assay is considerably higher than this value ***(29)***. If in doubt about the optimal antibiotic concentration for tolerance and persistence assays, test a range of concentrations and choose one that is so high that the killing rate does not increase anymore with increasing drug concentration. When comparing the antibiotic killing dynamics of different bacterial mutants or strains, measure the MIC of each of them. If they are very different, consider for your experiments that not only differences in antibiotic tolerance but also different intrinsic drug resistance (as expressed by MIC) can change the dynamics of bacterial killing in antibiotic treatment assays. See Table 4 for reference MIC values of *E. coli* K-12 MG1655 and *P. aeruginosa* PAO1.
16. Treating with 10 µg/mL ciprofloxacin, a very high concentration (ca. 1.000x MIC), is necessary to avoid artefacts caused by secondary killing of antibiotic-tolerant bacteria due to the induction of prophages ***(8)***.
17. To increase accuracy when pipetting small pellets and to reduce the risk of disturbing the pellet, one can fit a regular 200 µL pipette tip on top of a 1.000 µL tip. Like this, the large volume pipetted during washing can still be removed efficiently but the risk to wash out the small and sometimes invisible pellets at the bottom of the tube is reduced.

18. The pellets from samples of late-exponential-phase cultures treated with β-lactams are often difficult to homogeneously resuspend due to fulminant bacterial lysis and aggregation of cell debris. Vortexing the tubes for 15-30 seconds and pipetting the pellets up and down a few times are sufficient to reproducibly release the surviving bacteria into the PBS, because most of the hard pellets itself is merely composed of dead cell debris.
19. Bacteria treated with ciprofloxacin or tobramycin can take more than 36 h to form visible colonies on LB agar plates, in particular in case of *P. aeruginosa*. In order to include all bacterial survivors, it is therefore important to incubate the LB agar plates for 40-48 h in total at 37 °C before the final CFU counts are recorded.
20. The hallmark of antibiotic persistence is a time kill curve with a biphasic trajectory ***(2)***. In cases where it is necessary to distinguish between antibiotic tolerance and persistence, it is therefore important to verify biphasic killing by determining viable CFU/mL not only after 5 h of drug treatment but also after, e.g., 1 h, 3 h, and 7 h in order to record the dynamics of bacterial killing (like in a regular time kill curve). Previous work found that bacterial killing under conditions similar to those presented in this protocol is largely biphasic ***(8, 18)***.
21. The results presented in Fig. 3 highlight a few important aspects. First, the overall shape of the tolerance curves along the growth phases are very similar for both *E. coli* K-12 MG1655 and *P. aeruginosa* PAO1 Δ*pel* Δ*psl*, suggesting that the underlying phenomena causing antibiotic tolerance are similar irrespective of the model organism investigated (similar to previous results in ***(30)***). Second, it is well visible how the initially higher tolerance levels (up to 2-3 h of cultivation) are a consequence of stationary phase carryover and drop as more bacteria exit lag phase ***(8)***. Third, once bacteria start to successively enter stationary phase, the levels of tolerant cells

increase again. For both organisms, tobramycin tolerance is only observed in stationary phase. The dynamics of ampicillin and ciprofloxacin tolerance are largely parallel except for the exponential phase of *E. coli* (ca. 3–6 h after subculturing) where the stark decrease in ampicillin-tolerant cells is not mirrored by the levels of ciprofloxacin-tolerant cells.

22. The results presented in Fig. 4 reveal a massive presence of antibiotic-tolerant cells for both *E. coli* K-12 MG1655 and *P. aeruginosa* PAO1 Δ*pel* Δ*psl* in the stationary phase. The biphasic kill curve resulting from ciprofloxacin treatment reveals a large fraction of persisters and at the same time indicates that most bacteria in culture are still active in DNA processing (as a prerequisite for gyrase poisoning by fluoroquinolones). Whereas the complete tobramycin tolerance of both organisms can be explained by reduced drug uptake due to lowered membrane potential, the total tolerance observed against β-lactams suggests absence of actively dividing cells in the later stages of stationary phase under these conditions ***(11,31,32)***.

Table 4. MIC values of *E. coli* K-12 MG1655 and *P. aeruginosa* PAO1 Δ*pel* Δ*psl* in M9Glc medium. This table presents the minimum inhibitory concentration (MIC) values of *E. coli* K-12 MG1655 and *P. aeruginosa* PAO1 *Δpel Δpsl* in M9Glc as determined by the authors for the antibiotics used in this study. The use of different β-lactam antibiotics for *E. coli* and *P. aeruginosa* is addressed in **Note 9**. The values reported are the result of three independent biological replicates. N.D. = not determined

	Tobramycin	Ciprofloxacin	Ampicillin	Meropenem
Escherichia coli K-12 MG1655	0.25 μg/mL	0.015 μg/mL	3 μg/mL	N.D.
Pseudomonas aeruginosa PAO1 *Δpel Δpsl*	1 μg/mL	0.06 μg/mL	N.D.	0.5 μg/mL

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Figures and figure captions

Figure 1. Serial dilutions and spotting. This figure is a schematic representation of the serial dilutions performed in 96-well microtiter plate (left) and the transfer of all dilutions onto a dry LB agar plate in a square Petri dish for colony outgrowth (right). Briefly, 100 µL of each sample are transferred into the wells of row A after the wells in the other rows (B-H) have been filled with each 180 µL of sterile PBS. Serial dilutions (1:10) are performed with a multichannel pipet by transferring 20 µL from one row to the next (as indicated by arrows; left), mixing, discarding the tips, and then repeating this process until row H (dilution 10^{-8}) is reached. Subsequently, 10 µL spots of all dilutions are transferred onto the LB agar plate from the highest dilutions to the undiluted samples using a single set of tips (rows H to A, arrows 1-8).

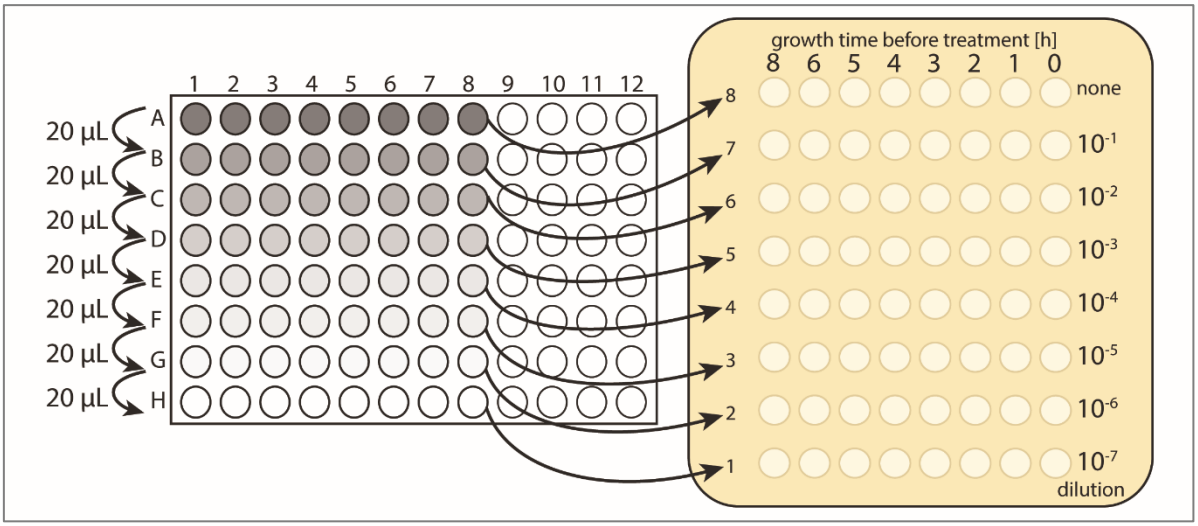
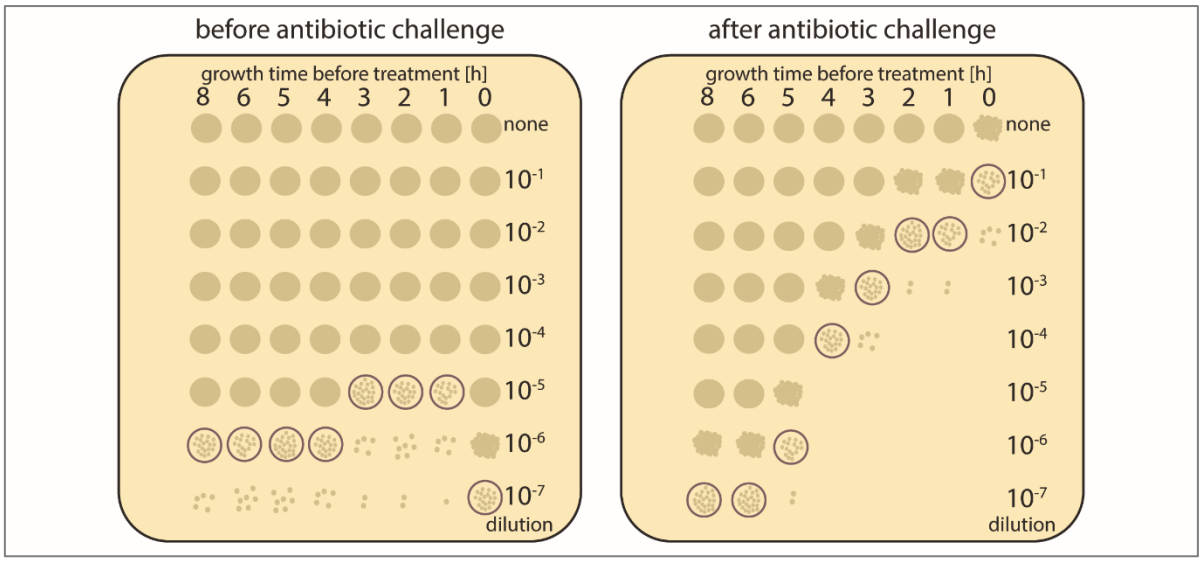


Figure 2. Colony counts after CFU regrowth. This figure is a schematic representation of plates from a typical experiment of treatment of bacteria along the growth curve after



colonies have grown up for 24–48 h. Regions containing between 10 and 100 colonies are highlighted by a violet circle and should be used for CFU/mL calculation.

Figure 3. Antibiotic tolerance of *E. coli* and *P. aeruginosa* along the growth phases. (A) Levels of *E. coli* K-12 MG1655 cells tolerant to 100 µg/mL ampicillin (light blue), 10 µg/mL ciprofloxacin (green), or 40 µg/mL tobramycin sulfate (red) were recorded throughout the bacterial growth phases from inoculation to early stationary phase. **(B)** Levels of *P. aeruginosa* PAO1 Δ*pel* Δ*psl* cells tolerant to 12 µg/mL meropenem (dark blue), 10 µg/mL ciprofloxacin (green), or 40 µg/mL tobramycin sulfate (red) were recorded throughout the bacterial growth phases from inoculation to early stationary phase. Results of one representative experiment are shown. The limit of detection (100 CFU/mL) is indicated by the upper edge of the grey bar.

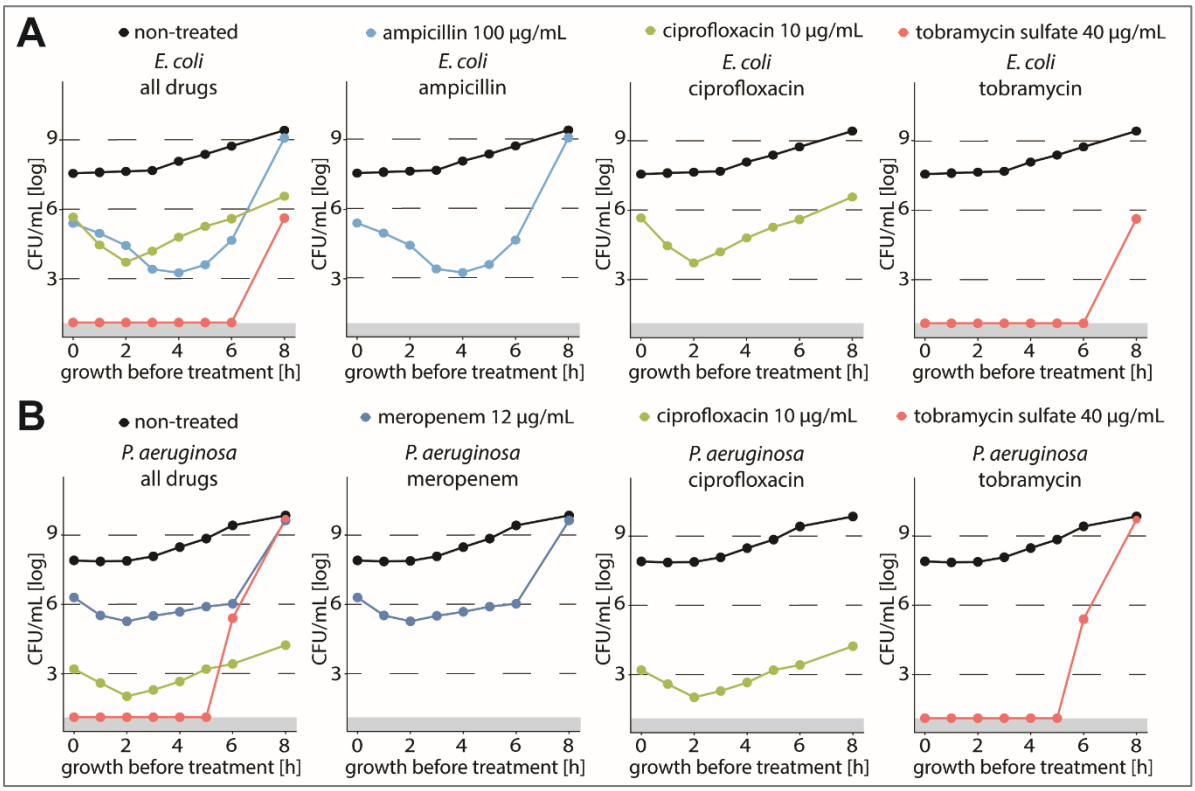


Figure 4. Antibiotic persistence of *E. coli* and *P. aeruginosa* in stationary phase. (A) A stationary-phase culture of *E. coli* K-12 MG1655 was treated with 100 µg/mL ampicillin (light blue), 10 µg/mL ciprofloxacin (green), or 40 µg/mL tobramycin sulfate (red) and bacterial survival was recorded over time. **(B)** A stationary-phase culture of *P. aeruginosa* Δ*pel* Δ*psl* was treated with 12 µg/mL meropenem (dark blue), 10 µg/mL ciprofloxacin (green), or 40 µg/mL tobramycin sulfate (red) and bacterial survival was recorded over time. Data points represent average values of three biological replicates and error bars show the standard error of the mean. The limit of detection is 100 CFU/mL (not shown).