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Population and Single-Cell Analysis of Antibiotic Persistence in *Escherichia coli*

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Abstract

Antibiotic persistence refers to the capacity of small bacterial subpopulations to transiently tolerate high doses of bactericidal antibiotics. Upon bactericidal antibiotic treatment, the bulk of the bacterial population is rapidly killed. This first rapid phase of killing is followed by a substantial decrease in the rate of killing as the persister cells remain viable. Classically, persistence is determined at the population level by time/kill assays performed with high doses of antibiotics and for defined exposure times. While this method provides information about the level of persister cells and the killing kinetics, it fails to reflect the intrinsic cell-to-cell heterogeneity underlying the persistence phenomenon. The protocol described here combines classical time/kill assays with single-cell analysis using real-time fluorescence microscopy. By using appropriate fluorescent reporters, the microscopy imaging of live cells can provide information regarding the effects of the antibiotic on cellular processes, such as chromosome replication and segregation, cell elongation, and cell division. Combining population and single-cell analysis allows for the molecular and cellular characterization of the persistence phenotype.

Introduction

This protocol aims to analyze the bacterial persistence phenotype in response to specific antibiotic treatment at the single-cell and population levels. Persistence describes the capacity of small subpopulations within an isogenic population to endure high doses of bactericidal antibiotics (fluoroquinolones, aminoglycosides, β-lactams, etc.), with the minimal inhibitory concentration (MIC) of the so-called persister cells being identical to that of the bulk of the population. Biphasic killing dynamics, when measuring bacterial survival over time in the presence of an antibiotic, reveal the presence of transiently drug-tolerant cells, with an initial rapid eradication of the non-persister cells, followed by a much slower killing rate of the persister cells. Upon antibiotic removal, these cells give rise to a genetically identical population that displays similar killing dynamics when treated with the same antibiotic[1,2]. In contrast to

persistence, antibiotic resistance is defined at the population level and is generally a consequence of either *de novo* mutations or the horizontal gene transfer of a resistance-conferring plasmid[3]. While the mutations responsible for resistance are mostly located in the target of the drug or in the promotor regions of the drug efflux pumps, genes altering the persistence frequency identified by genome-wide and targeted mutant analysis approaches have proven to be numerous and diverse[2,3,4,5,6,7,8]. Therefore, it is likely that bacterial cells can enter the persister state through multiple pathways[9,10,11], and approaches to investigate the persistence phenomenon at the single-cell level are needed to characterize the physiology of these persister cells.

The recent development of microfluidic tools used in combination with fluorescence microscopy has paved the way for the characterization of the persistence phenotype and highlighted the role of key cellular processes, such as chromosome replication[12], DNA repair[13], and cell division[14], in persister cell formation. In this paper, we describe an integrated approach combining classical microbiology assays with single-cell live imaging to characterize persister cells generated in exponentially growing *Escherichia coli* cultures treated with a high dose of ofloxacin. The protocol described here can be applied to study the antibiotic persistence phenomenon in other bacterial species, such as *Bacillus subtilis*[15], or conditions (e.g., antibiotic persistence following β-lactam treatment[16]) and can easily be modified to investigate the many phenomena involving phenotypic heterogeneity[17,18,19]. Furthermore, the setup described in this paper can be combined with other fluorescent reporters to investigate distinct cellular parameters of interest, such as intracellular levels of pH[20] or ATP[21] at the single-cell level, which may potentially produce novel insights into the antibiotic persistence phenomenon.

Protocol

NOTE: Use sterile culture glassware, pipette tips, and growth medium. Here, *E. coli* cells were grown in a low-autofluorescence chemically defined medium (see **Table of Materials**). Inoculations were performed in the presence of a Bunsen burner to minimize the risk of contamination.

1. Cell culture and growth curve

1. Streak the strain of interest from a frozen glycerol stock on a Luria-Bertaini (LB) agar plate (supplemented with a selective antibiotic, if required), and incubate at 37 °C overnight (between 15 h and 19 h) to obtain single colonies.

   NOTE: The experiment presented here uses two strains, *E. coli* K-12 MG1655 corresponding to the *wt* strain and the isogenic MG1655 *hupA-mCherry* strain[22]. The latter strain expresses the fluorescence-tagged α-subunit of the HU nucleoid-associated protein. The *hupA-mCherry* reporter is integrated at the native *locus* of *hupA*. HU-mCherry serves as a proxy to follow the nucleoid dynamics in live cells as it binds to DNA in a non-specific manner.

2. Inoculate 5 mL of medium (here, 3-[N-morpholino] propanesulfonic acid-based medium [MOPS]; **Table 1**, **Table 2**, and **Table 3**) supplemented with glucose 0.4% and a selective antibiotic (if required) with an isolated colony in a glass tube (≥25 mL), and place the tube in a shaking incubator set to 37 °C and 180 rotations per minute (rpm) overnight (between 15 h and 19 h).

Alternatively, plastic tubes, glass, or plastic flasks (≥ 25 mL) can be used instead of glass tubes.

NOTE: MOPS medium supplemented with glycerol at a final concentration of 0.4% was used throughout the experiments described in this paper, except for the overnight cultures which were performed in MOPS glucose 0.4%. The generation time of *E. coli* in MOPS supplemented with glucose is shorter than that in MOPS glycerol. Using MOPS glucose 0.4% instead of MOPS glycerol 0.4% at this step ensures that the cells reach the stationary phase within 19 h. Other growth media, such as M9 or rich defined medium (RDM) supplemented with distinct carbon sources, can also be used. It should, however, be noted that the growth rate and the persistence frequency are different depending on the medium and/or the carbon used[23].

3. The following morning, centrifuge 1 mL of culture at 2,300 x *g* for 3 min, discard the supernatant, and gently resuspend the pellet in the same volume of phosphate-buffered saline (PBS). Measure the optical density at 600 nm ($OD_{600\ nm}$), and calculate the volume needed for an initial $OD_{600\ nm}$ of 0.01 in a final volume of 2 mL.

4. Place 2 mL of MOPS glycerol 0.4% medium into a well of a clear bottom 24-well plate, and inoculate with the calculated overnight culture volume. Place the 24-well plate in an automated microplate reader (see **Table of Materials**) to monitor the $OD_{600\ nm}$ for 24 h. Set the microplate reader to measure the $OD_{600\ nm}$ every 15 min at a temperature of 37 °C and with high orbital rotation (140 rpm).

   NOTE: If the strain used in the experiment encodes a fluorescent reporter, make sure that its growth rate is comparable to that of the *wt* to avoid any artifact in the subsequent experiments, as the growth rate affects the antibiotic persistence frequency[23]. Due to detection limitations at very low cell densities and the potential strain-specific effects on the $OD_{600\ nm}$/colony forming units (CFU) relationship, it is recommended to evaluate the growth kinetics by monitoring the $CFU \cdot mL^{-1}$ when working with uncharacterized strains.

2. Determination of the minimal inhibitory concentration of the antibiotics

NOTE: The minimum inhibitory concentration (MIC) is defined as the lowest dose of antibiotic at which no bacterial growth is observed. The determination of the MIC needs to be performed for each antibiotic and strain. In the experiments described here, the fluoroquinolone antibiotic ofloxacin (OFX) was used. The determination of the MIC allows for confirmation that the antibiotic solution has been correctly prepared, the antibiotic is active, and the strains are equally sensitive to the antibiotic. Here, the published agar dilution method was performed to determine the MIC to OFX of the different strains used[24]. The MIC of a given antibiotic to a given bacterial strain can also be determined *via* the broth dilution method[24].

1. Preparation of the plates for MIC determination

   1. Prepare a master stock solution for the antibiotic used in the experiments by dissolving 5 mg of OFX in 1 mL of ultrapure water. Add 20 µL of 37% HCl to increase the solubility of the OFX.

   2. Melt 100 mL of sterile LB agar, and keep it at 55 °C to avoid solidification. Prepare six small glass flasks (25 mL), and pipet 5 mL of liquid LB agar medium into each flask using a sterile pipette.

   3. Dilute 10 µL of the 5 $mg \cdot mL^{-1}$ OFX master stock solution in 90 µL of ultrapure water (1:10 dilution

of the master stock).Add 0 µL, 2 µL, 4 µL, 6 µL, 8 µL, or 10 µL of the 500 µg·mL^{-1} OFX solution to each of the six glass flasks containing 5 mL of LB agar medium to generate LB agar medium with final concentrations of 0 µg·mL^{-1}, 0.02 µg·mL^{-1}, 0.04 µg·mL^{-1}, 0.06 µg·mL^{-1}, 0.08 µg·mL^{-1}, and 0.1 µg·mL^{-1} OFX, respectively. Mix the solution by rotating the flasks several times.

NOTE: If the MIC of the antibiotic of interest is known, the range of concentrations should reach from below to above the actual MIC. If the MIC is unknown, a large range of concentrations with a $\log_2$ dilution series is recommended. Make sure that the LB agar medium has cooled down before adding the antibiotic, as high temperatures may inactivate it. Nevertheless, it is important not to let the LB agar medium solidify before adding the antibiotic, as this could lead to the non-homogenous distribution of the antibiotic in the LB agar medium.

4. Pour 5 mL of each of the six LB agar media prepared in step 2.1.3 in a dose-increasing manner into a 6-well culture plate using a sterile pipette. Let the agar cool down until solidification, and dry the plate before using.

   NOTE: Prepare the antibiotic solution, and the culture plate the day the assay is performed.

2. MIC determination assay

   1. Inoculate 5 mL of LB medium with an isolated colony in a glass tube (≥25 mL), and place the glass tube in a shaking incubator set to 37 °C and 180 rpm overnight (between 15 h and 19 h).

   2. The following morning, measure the $OD_{600\ nm}$, and dilute the culture into a test tube to a final cell density of 1 x 10^7 CFU·mL^{-1} in PBS. For the strains tested here, 1 x 10^7 CFU·mL^{-1} corresponds to an $OD_{600\ nm}$ of 0.0125.

      NOTE: If the correlation between the CFU·mL^{-1} and $OD_{600\ nm}$ is not known, the growth curve determined by CFU and $OD_{600\ nm}$ measurements should be established to calculate the correlation factor between the CFU·mL^{-1} and $OD_{600\ nm}$.

   3. Spot 2 µL of the previously diluted culture onto every well of the dried 6-well plate. Let the spots dry before placing the plate into an incubator at 37 °C overnight (between 15 h and 19 h).

   4. The next day, count the colonies formed in each well. The MIC corresponds to the well with the minimum concentration of antibiotic where no bacterial growth is detected.

3. Spot assay

NOTE: The spot assay method is a qualitative approach that allows the estimation of the number of viable cells (cells able to generate colonies after antibiotic stress). The spot assay is performed prior to the time-kill assay to provide insights into the viability of the strain used in the conditions tested and to inform about the dilutions needed during the time-kill assay (see section 4).

1. To prepare the LB agar plates for spot assays, pour 50 mL of LB agar into a square Petri dish (144 cm^2). Prepare one square Petri dish per time point. Let the LB agar solidify, and dry the plates before use.

2. Inoculate 5 mL of medium (MOPS glucose 0.4%) with an isolated colony in a glass tube (≥25 mL), and place the

tube in a shaking incubator set to 37 °C and 180 rpm overnight (between 15 h and 19 h).

3. The next day, measure the $OD_{600\ nm}$, and dilute the culture into fresh temperature-adjusted medium (37 °C, MOPS glycerol 0.4%) in a glass tube (≥25 mL) to a final $OD_{600\ nm}$ of ~0.001. Let the culture grow overnight in an incubator at 37 °C at 180 rpm (between 15 h and 19 h).

4. On the following day, measure the $OD_{600\ nm}$, and incubate the culture to a final $OD_{600\ nm}$ of 0.3.

5. While incubating, prepare 96-well plates by placing 90 µL of 0.01 M $MgSO_4$ solution in every well except the wells of the first row (row A). The 96-well plates will be used for 10-fold serial dilution in step 3.7.

   NOTE: In this experiment, two strains (*wt* and *hupA-mCherry* strain) were tested in triplicate at seven different time points. As one plate comprises 12 columns, one plate can be used for two time points, and, thus, a total of four plates were prepared.

6. At an $OD_{600\ nm}$ of 0.3, withdraw 200 µL of each bacterial culture. These samples correspond to the t0 (untreated) timepoint prior to the antibiotic treatment and allow the determination of the $CFU \cdot mL^{-1}$ before the antibiotic treatment.

7. Centrifuge the samples at 2,300 x *g* for 3 min. During the centrifugation time, add the desired concentration of OFX to the liquid culture, and continue to incubate at 37 °C while shaking.

   NOTE: Here, OFX was used at a concentration of 5 $\mu g \cdot mL^{-1}$ (corresponding to the MIC multiplied 83-fold). In a previous study, this concentration was used to characterize the persistence phenomenon under OFX exposure[25]. The antibiotic concentration used for the time/kill assays can vary depending on the antibiotic, the culture medium, and the state of bacterial growth.

8. After centrifugation, resuspend the cell pellet in 200 µL of 0.01 M $MgSO_4$ solution. Place 100 µL in the empty well of the 96-well plate prepared in step 3.5. Perform a dilution by transferring 10 µL of the well in row A into the well in row B containing 90 µL of 0.01 M $MgSO_4$. Continue the serial dilutions by transferring 10 µL of the well in row B into the well in row C. Repeat until reaching a dilution of 10^{-7} (with every transfer being a 10-fold dilution).

   NOTE: In this experiment, six cultures (three for the *wt* strain and three for the *hupA-mCherry* strain) were tested for every timepoint. According to the protocol, a muti-channel pipette is used to perform the serial dilutions for the six strains at the same time. To minimize the technical error, the pipette tips are changed between every transfer.

9. Spot 10 µL of each dilution onto the LB agar plates prepared in step 3.1.

   NOTE: A multi-channel pipette is used to simultaneously spot the same dilution (e.g., a 10^{-4} dilution) for the six cultures. During the spotting, the first stop of the multi-channel pipette should be used without pushing the second stop, as this can result in microdroplets being dispensed onto the plate.

10. At relevant time points after the antibiotic addition, withdraw 200 µL of the culture, and perform serial dilutions as described in step 3.8. Spot 10 µL of each dilution as described in step 3.9.

    NOTE: For the experiment described here, seven time points were collected (t0, t1h, t2h, t3h, t4h, t5h, t6h). For strains exhibiting increased antibiotic susceptibility

as compared to the *wt,* multiple washes in $MgSO_4$ 0.01 M can be performed to remove residual antibiotics.

11. Incubate the plates at 37 °C overnight (between 15 h and 19 h). The next day, count the number of colonies at the two highest dilutions for which colonies can be detected. Ideally, the spots on the agar plates for these dilutions contain between 3 to 30 colonies allowing the accurate determination of the $CFU \cdot mL^{-1}$ for each sample.

12. Calculate the survival ratio by dividing the calculated $CFU \cdot mL^{-1}$ of each timepoint by the $CFU \cdot mL^{-1}$ of the initial population at t0.

4. Time-kill assays

NOTE: While spot assays are an easy-to-use method to estimate the survival rate of a given strain for a given antibiotic, time-kill assays give a higher-resolution survival rate and are performed to accurately quantify the bacterial viability. The profile of the killing curve can be used to determine whether a given bacterial strain is sensitive, tolerant, or resistant to the antibiotic in a given condition. Moreover, time-kill assays allow the determination of the time of antibiotic exposure needed to detect the persistence phenomenon (beginning of the second slope of the biphasic killing curve) as well as the persistence frequency.

1. Prepare LB agar plates for the time-kill plating assay. Pour 25 mL of LB agar into a Petri dish (±57 cm^2). Prepare at least two Petri dishes per timepoint (two dilutions per timepoint per strain are plated).

2. Let the LB agar solidify, and dry the plates before adding five to eight sterile glass beads to each plate. Invert and label the plates according to the strain/condition/timepoint.

NOTE: Glass beads allow the spreading of bacteria on the agar plates during step 4.7 and step 4.9. Alternatively, the cells can be dispersed on the agar plate using a spreader.

3. For each sample, prepare 10-fold serial dilution glass tubes containing 900 µL of 0.01 M $MgSO_4$ solution. The number of dilution glass tubes per sample that needs to be prepared corresponds to the dilutions needed to detect colonies in the spot assay.

4. Inoculate 5 mL of medium (MOPS glucose 0.4%) with an isolated colony in a glass tube (≥25 mL), and place the tube in a shaking incubator set to 37 °C and 180 rpm overnight (between 15 h and 19 h).

5. After 16 h of growth, measure the $OD_{600\ nm}$, and dilute the culture into fresh temperature-adjusted medium (37 °C, MOPS glycerol 0.4%) in a glass tube to a final $OD_{600\ nm}$ of ~0.001. Let the culture grow overnight in an incubator at 37 °C at 180 rpm (between 15 h and 19 h).

6. On the following day, measure the $OD_{600\ nm}$, and incubate the culture to a final $OD_{600\ nm}$ of 0.3.

7. At an $OD_{600\ nm}$ of 0.3, withdraw 100 µL of the culture, dilute according to the data obtained in the spot assay (at an $OD_{600\ nm}$ of 0.3 and without antibiotic, a 10^{-5} dilution usually gives 200-300 colonies), and plate 100 µL on the LB agar plates prepared in steps 4.1-4.2. Gently shake the plates to avoid the beads contacting the dish edges, as this can lead to a non-homogenous spreading of the bacterial cells on the LB agar medium. This first sample prior to the antibiotic treatment corresponds to the t0 time point ($CFU \cdot mL^{-1}$ before antibiotic treatment).

8. Add the desired concentration of OFX, and continue to incubate at 37 °C while shaking.

NOTE: Here, OFX was used at a concentration of 5 $\mu g \cdot mL^{-1}$ (corresponding to the MIC multiplied 83-fold).

9. At relevant time points after the antibiotic addition, withdraw 100 µL of the culture, dilute according to the data obtained in the spot assay, and plate 100 µL on the LB agar plates prepared in steps 4.1-4.2. Plate the cells as described in step 4.7.

   NOTE: For the experiment described here, seven time points were collected (t0, t1h, t2h, t3h, t4h, t5h, t6h). For strains exhibiting an increased antibiotic susceptibility as compared to the *wt*, multiple washes in $MgSO_4$ 0.01 M can be performed to remove residual antibiotics.

10. Incubate the plates at 37 °C overnight (between 15 h and 19 h). The next day, count the number of colonies at the two highest dilutions for which colonies can be detected. Ideally, the plates should contain 30-300 colonies to allow an accurate determination of the $CFU \cdot mL^{-1}$ in each sample.

11. Calculate the survival ratio by normalizing the $CFU \cdot mL^{-1}$ at each time-point by the $CFU \cdot mL^{-1}$ at t0. Plot the $\log_{10}$ normalized $CFU \cdot mL^{-1}$ as a function of time.

5. Microfluidic time-lapse microscopy imaging

NOTE: The following section describes the preparation of the microfluidic plate as well as the time-lapse image acquisition and image analysis procedure. The aim of this experiment is to observe and analyze the persistence phenotype upon antibiotic treatment at the single-cell level. The data collected during this experiment can be used to generate a wide range of results depending on the question addressed and/or the fluorescent reporters used during the experiment. In the experiment described here, quantitative analysis of the cell length and HU-mCherry fluorescence[22], reflecting the nucleoid organization in persister and non-persister cells, was carried out.

1. Bacterial cell culture for microfluidic time-lapse microscopy

   1. Inoculate 5 mL of medium (MOPS glycerol 0.4%, supplemented with a selective antibiotic if required) with an isolated colony in a glass tube (≥25 mL), and place the tube in a shaking incubator set to 37 °C and 180 rpm overnight (between 15 h and 19 h).

   2. The next day, measure the $OD_{600\ nm}$, and dilute the culture in fresh temperature-adjusted medium (37 °C, MOPS glycerol 0.4%) in a glass tube to a final $OD_{600\ nm}$ of ~0.001. Let the culture grow overnight (between 15 h and 19 h) in a shaking incubator at 37 °C and 180 rpm to obtain an early exponential-phase culture on the next day.

2. Preparation of the microfluidic plate and time-lapse microscopy imaging

   NOTE: Microfluidic experiments can be performed in commercially available microfluidic devices (as described here) or in in-house produced microfluidic systems.

   1. Remove the conservation solution (if present) from every well of the microfluidic plate, and replace it with a fresh culture medium.

      NOTE: If the microfluidic plate contains a waste outlet well, the conservation solution of the outlet well should be removed but not replaced by the medium.

   2. Seal the microfluidic plate with the manifold system by clicking on the **Seal** button or through the

microfluidic software (first select **Tool**, followed by **Seal Plate**).

NOTE: To seal the plate, a uniform pressure should be applied to the plate and the manifold by manually squeezing the plate against the manifold. If performed correctly, the note "sealed" should appear on the ONIX2 interface. It is important to not apply any pressure onto the glass slide to avoid any potential risk of breaking the manifold.

3. Once sealed, perform a first priming sequence (click on **Run Liquid Priming Sequence** on the microfluidic software interface).

   NOTE: **Run Liquid Priming Sequence** corresponds to 5 min of perfusion at 6.9 kPa for wells 1-5, followed by 5 min of perfusion at 6.9 kPa for well 8, and a final perfusion round for well 6 for 5 min at 6.9 kPa. The **Run Liquid Priming Sequence** allows the removal of the conservation solution that may still be present in the channels connecting the different wells.

4. Incubate the plate in a thermostatically controlled cabinet of the microscope at the desired temperature (here, 37 °C) for a minimum of 2 h before the start of the microscopy imaging.

5. Start a second **Run Liquid Priming Sequence** before beginning the experiment.

6. Seal off the microfluidic plate by clicking on Seal off on the microfluidic software interface. Replace the medium in well 1 and well 2 with 200 µL of fresh medium, in well 3 with 200 µL of fresh medium containing the antibiotic (here, OFX at 5 $\mu g \cdot mL^{-1}$), in well 4 and well 5 with 200 µL of fresh medium, in well 6 with 200 µL of fresh medium, and in well 8 with 200 µL of the culture sample (from step 5.1.2) diluted to an $OD_{600\ nm}$ of 0.01 in the fresh medium.

7. Seal the microfluidic plate as described in step 5.2.2, and place the plate onto the microscope objective inside the microscope cabinet.

   NOTE: Make sure to place a drop of immersion oil onto the microscope objective before placing the microfluidic plate.

8. In the microfluidic software, click on **Cell Loading** to allow the cell loading into the microfluidic plate.

   NOTE: The **Cell Loading** step comprises 15 s of perfusion at 13.8 kPa for well 8, followed by 15 s of perfusion at 27.6 kPa for well 6 and well 8, and a final perfusion round for well 6 for 30 s at 6.9 kPa. The density of the cells in the microfluidic plate is critical for the experiment. The first part of this microfluidic protocol consists of growing bacteria for 6 h in a fresh medium before the antibiotic treatment. After 6 h of growth, the cell density has to be sufficient to detect the rare persister cells (in the conditions used in this study, the persister cells generate at a frequency of 10^{-4}). If the cell density is too high, it is difficult to distinguish the individual cells, which prevents precise single-cell analysis. As the growth rate is directly dependent on the medium, the density of cells in the microscopy fields should be assessed before launching the experiment.

9. Set an optimal focus using transmitted light mode, and select several regions of interest (ROIs) where an appropriate cell number is observed (up to 300 cells per field).

   NOTE: Select at least 40 ROIs to make sure that the rare persister cells are imaged.

10. On the microfluidic software, click on **Create a Protocol**. Program the injection of fresh medium at 6.9 kPa for 6 h (well 1-2), followed by the injection of the medium containing the antibiotic at 6.9 kPa for 6 h (well 3), and, finally, the injection of fresh medium at 6.9 kPa for 20 h (wells 4-5).

    NOTE: A bacterial culture diluted to an $OD_{600\ nm}$ of 0.01 allows bacteria to grow in the microfluidic chamber for 6 h, ensuring that the cells are in the exponential growth phase. Depending on the number of cells introduced into the microfluidic device during the loading step (see step 5.2.8), the duration of the growth phase can be adapted to obtain up to 300 cells per ROI. As persistence is a rare phenomenon, increasing the number of cells per ROI improves the possibility of observing persister cells. The cell number should, however, not exceed 300 cells per ROI, as this makes single-cell analysis tedious.

11. Perform microscopy imaging in time-lapse mode with one frame every 15 min using transmitted light and the excitation light source for the fluorescent reporter. Here, a 560 nm excitation light source for the mCherry signal was used (580 nm LED at 10% power with filter 00 [530-585 ex, 615LP em, Zeiss] and 100 ms exposure for mCherry). The Zeiss-compatible Zen3.2 software was used for cell imaging.

3. Image analysis

    NOTE: The opening and visualization of the microscopy images are performed with the open-source ImageJ/Fiji software (https://fiji.sc/)[26]. The quantitative image analysis is performed using the open-source ImageJ/Fiji software and the free MicrobeJ plugin (https://microbej.com)[27]. In this protocol, the MicrobeJ 5.13l(14) version was used.

    1. Open the ImageJ/Fiji software on the computer, and drag the hyperstack time-lapse microscopy images into the Fiji loading bar. Use **Image > Color > Make Composite** to fuse the different channels of the hyperstack. If the channels of the time-lapse experiment do not correspond to the desired color (e.g., the phase contrast is shown in red instead of gray), use **Image > Color > Arrange Channels** to apply the appropriate color to the channels.

    2. Open the MicrobeJ plugin, and detect the bacterial cells using the manual editing interface. Delete the automatically detected cells, and manually outline the persister cells of interest frame by frame.

        NOTE: Different settings can be used to automatically detect individual cells. Manual detection was used here as the analyzed persister cells form long filaments, which are rarely detected correctly using automatic detection.

    3. After detection, use the **Result** icon in the MicrobeJ manual editing interface to generate a ResultJ table. Save the ResultJ file, and use the ResultJ table to gain insights into different parameters of interest of the single-cell analysis. In the case of this protocol, the mean fluorescence of the HU-mCherry intensity, the cell length, and the cell area of individual cells were exported.

Representative Results

As described above, the strains used for the single-cell phenotypic analysis of persister cells were characterized in MOPS glycerol 0.4% medium. The monitoring of the OD_{600nm} over time showed no difference between the *wt* and *hupA*-

mCherry strains (**Figure 1**). This indicates that the expression of the HU-mCherry fusion protein did not impact growth in these conditions. The bacterial cells of both strains initially inoculated at an $OD_{600\ nm}$ of 0.01 reached the exponential phase ±8 h after inoculation.

The MIC of OFX was determined by standardized methods (here, serial agar dilution)[24]. MIC is defined as the minimal concentration where no visible growth is detected. The MIC of OFX for both strains was determined to be 0.06 $\mu g \cdot mL^{-1}$, indicating that the *hupA-mCherry* fusion had no effect on the sensitivity to OFX in comparison with the isogenic *wt* strain (**Figure 2**).

We further determined the effect of a lethal OFX treatment (83-fold MIC) on the viability of both strains used in this study. As the viable cell count decreases over time with OFX exposure, the dilutions of bacterial cultures need to be adjusted appropriately to reach 30 to 300 colonies per plate. To determine the appropriate dilutions over time, a spot assay was performed, where 10 µL from 0 to 10^{-7} serial 10-fold dilutions were placed on square Petri dishes using a multi-channel pipette. The appropriate dilutions were those where isolated clones were visible (e.g., at t0 = 10^{-5}, t1h = $10^{-4}/10^{-3}$, t4h = $10^{-2}/10^{-1}$) (**Figure 3**).

While the spot assay is an easy method to gain insights into the kinetics of OFX-mediated killing, it fails to accurately determine the killing dynamics. When the viability of exponentially growing cells treated with OFX was monitored by the time-kill assay, a typical biphasic curve was observed (**Figure 4**). The first slope of the curve reflects the rapid killing of the non-persister population (red dashed line). In the conditions tested here, up to 99.9% of the cells were unable to form colonies after 3 h in the presence of OFX. This first phase of killing is followed by a second phase, showing a slower killing rate (blue dashed line), which reveals the presence of drug-tolerant persister cells. In the conditions tested, the persister phase started around 3 h after the OFX addition, highlighting the necessity to expose the cells to OFX for longer than 3 h to investigate the persister phenotypes. Importantly, the time-kill curve shows that the *hupA-mCherry* fusion protein had no effect on the time-kill kinetics. The strain encoding the translational fluorescent fusion can, therefore, be used to monitor the persister cells using fluorescence microscopy.

We further went on to investigate the persistence phenomenon at the single-cell level. To do so, the *hupA-mCherry* strain was introduced into a microfluidic plate, which allowed for the change of medium conditions (here, growth, treatment, and recovery) while performing time-lapse microscopy on a given ROI. During the first step of the microfluidic experiment, the cells introduced into the microfluidic device were perfused with growth medium (MOPS glycerol 0.4%) and divided with a generation time of ~2 h (**Figure 5** and **Figure 6**). This first phase of growth indicates that cells were viable and actively dividing before the OFX treatment.

After this first phase of growth, the cells were perfused with growth medium supplemented with 5 $\mu g \cdot mL^{-1}$ OFX for 6 h. As soon as the antibiotic reached the cells, cell division was blocked (**Figure 5** and **Figure 6**). After 6 h of OFX treatment, the cells were perfused with fresh medium. While the vast majority of the cells were unable to resume growth (**Figure 5** and **Figure 6**), a small subpopulation of bacteria was capable of elongating and generating filamentous cells[25]. These cells, which were able to divide and generate viable daughter cells after the OFX treatment, can be defined as the persister cells.

As this setup allows for the visualization of the persister cells before, during, and after treatment, it not only provides information about the persister phenotype during the recovery phase but also about the physiological state of the persister cells before the treatment (**Figure 6**). In the conditions tested, the persister cells divided similarly to non-persister cells prior to the OFX treatment, indicating that the observed persister cells did not originate from a dormant subpopulation (**Figure 6**)[25].

The cell length analysis of persister cells during the recovery phase revealed that each filament had a specific rate of elongation. The cell length reached by each persister before the first division differed from one persister to another. Similarly, the timing of the first division event was highly heterogeneous (**Figure 6**). The dividing persister filament generated multiple daughter cells, which started to grow and divide, for the most part, similarly to untreated cells (**Figure 7**). The successive division of the filament then resulted in a progressive decrease in the cell length, ultimately giving rise to daughter cells with similar cell length to before the OFX treatment (**Figure 6** and **Figure 7B**). The vast majority of the cells were unable to induce filamentation after OFX removal. This large cell population corresponds to the dead cells (**Figure 5** and **Figure 6**).

The fluorescent fusion of the nucleoid-associated protein HU allows for the visualization of the dynamics of the nucleoid[22]. The analysis of the total fluorescence intensity of HU-mCherry within the cell can be used as a proxy for the DNA content[22,25]. During the growth phase (before OFX treatment), the total mCherry fluorescence intensity varied, reflecting the dynamics of chromosome replication and segregation during the cell cycle (**Figure 8**). After OFX addition, the mCherry fluorescence increased at the mid-cell, indicative of nucleoid compaction, which has been shown to be induced by the formation of double-strand DNA breaks[28] (**Figure 5**). Double-strand DNA breaks are a consequence of the mechanism of action of OFX, which corrupts the type II topoisomerases DNA-gyrase and topoisomerase IV[29,30]. In *E. coli*, DNA-gyrase is the primary target of OFX[29,30]. By binding its target at a critical step of the double-strand passage mechanism, OFX inhibits the relegation of the cleaved DNA strands, ultimately leading to the release of double-strand DNA breaks[30]. As described above, the persister cells to OFX treatment started to filament during recovery[25] (**Figure 6**). The increase in cell length correlated with an increase in the total mCherry fluorescence intensity, which reflects replication restart and an increase in the nucleoid abundance in the filament[25] (**Figure 7a** and **Figure 8**). For dead cells, the total mCherry fluorescence intensity remained stable during the treatment and during the recovery phase, indicating that these cells were unable to replicate their chromosomes after OFX removal (**Figure 8**). Microfluidic video (**Video 1**) of *E. coli* HU-mCherry cells before, during, and after ofloxacin treatment is also shown.

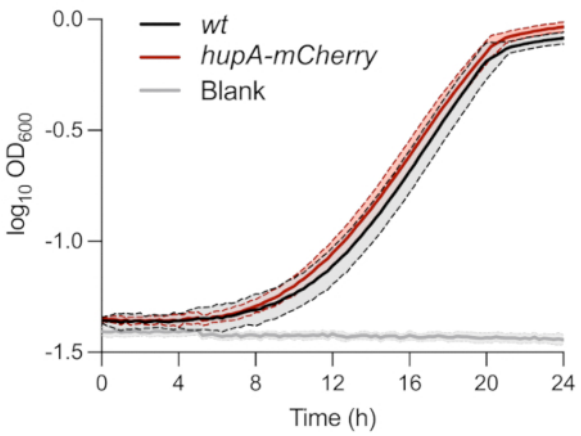

Figure 1: Growth monitoring of *wt* and *hupA-mCherry E. coli* strains. Optical density monitoring ($OD_{600\ nm}$) of *wt* (black) and *hupA-mCherry* (red). The shades and dashed lines indicate the standard deviations of the biological triplicates. Please click here to view a larger version of this figure.

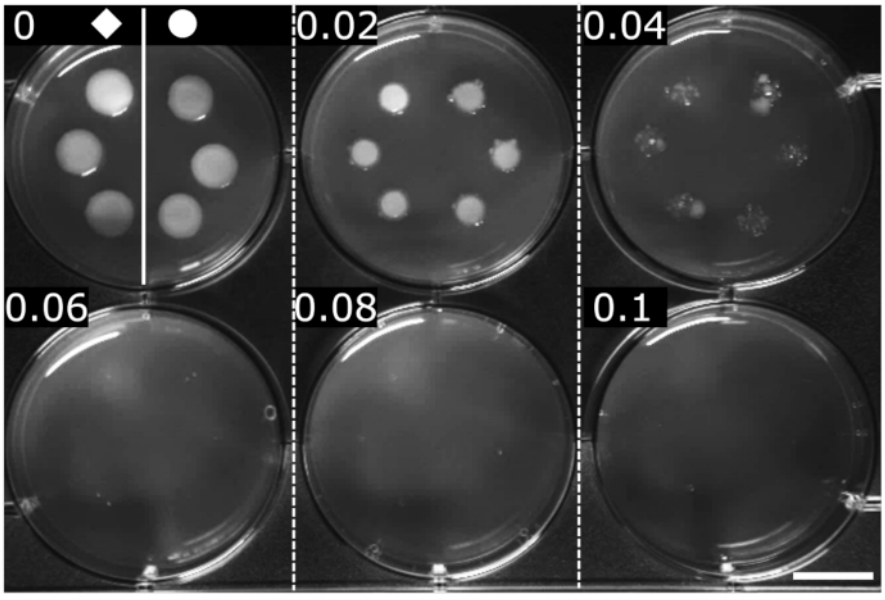

Figure 2: Determination of the MIC of OFX for the *wt* and *hupA-mCherry E. coli* strains. The *wt* (♦) and *hupA-mCherry* (●) were grown in LB medium, and 2 µL were spotted on serial dilutions of OFX-containing LB agar (concentration indicated in each panel in $\mu g \cdot mL^{-1}$). Growth inhibition is visible at a minimum of 0.06 $\mu g \cdot mL^{-1}$. The figure is a representative experiment of biological triplicates. Scale bar = 1 cm. Please click here to view a larger version of this figure.

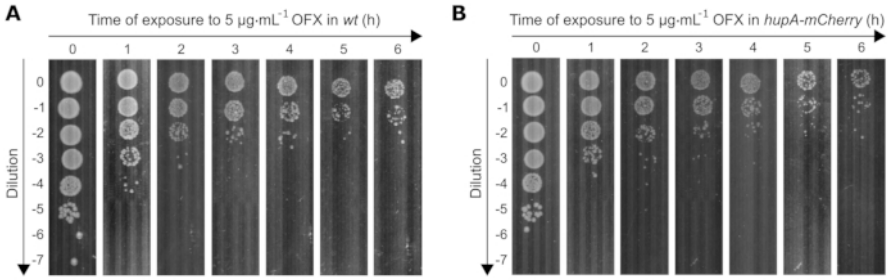

Figure 3: Spot assay of *wt* and *hupA-mCherry E. coli* strains upon exposure to OFX. The (**A**) *wt* and (**B**) *hupA-mCherry* strains were grown in MOPS glycerol 0.4% as described in the protocol (section 3), and the exponentially growing cells ($OD_{600\ nm}$ = 0.3) were treated with 5 µg·mL^{-1} OFX. T0 corresponds to the time point before the addition of OFX. T1, T2, T3, T4, T5, and T6 correspond to 1-6 h after the OFX addition. The figure is a representative experiment of biological triplicates. Please click here to view a larger version of this figure.

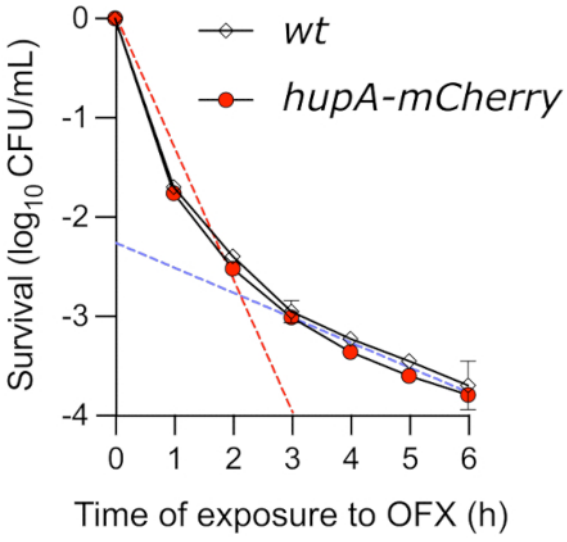

Figure 4: Time-kill assay of *wt* and *hupA-mCherry E. coli* strains upon exposure to OFX. The *wt* (♦) and *hupA-mCherry* (●) strains were grown in MOPS glycerol 0.4% as described in the protocol (section 4), and the exponentially growing cells ($OD_{600\ nm}$ = 0.3) were treated with 5 µg·mL^{-1} OFX. The dashed lines indicate the first "rapid" (red) killing phase and the second "slow" (blue) killing phase, corresponding to the sensitive and persistent subpopulations (obtained by linear regression between T0 and T2, as well as between T3 and T6, respectively). The error bars indicate the standard deviations of the biological triplicates. Please click here to view a larger version of this figure.

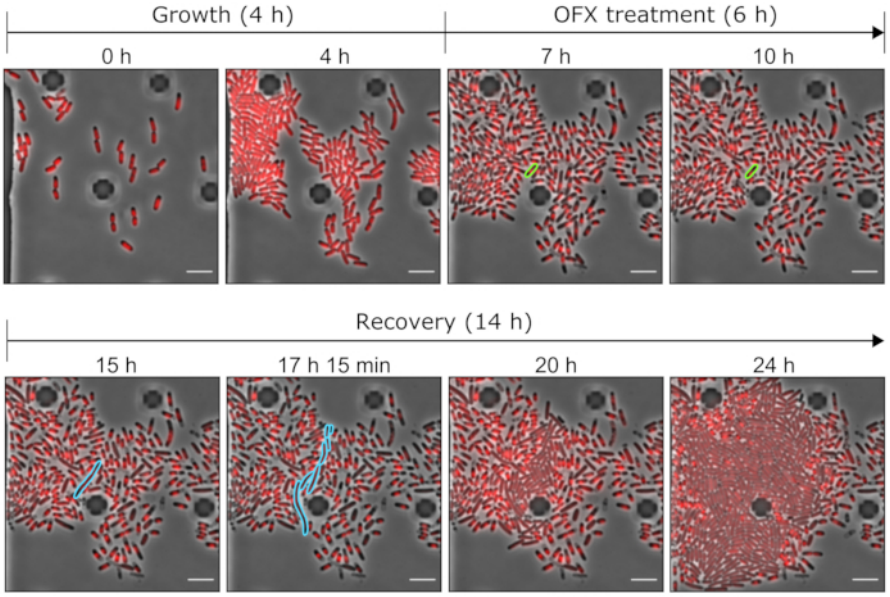

Figure 5: Representative images of the OFX persister and dead cells using microfluidic tools. Representative microscopy images showing the relevant time points of the microfluidic experiment performed with the *hupA-mCherry* strain (phase contrast in grey, HU-mCherry signal in red). The cells expressing the tagged *hupA-mCherry* were grown in a microfluidic plate (here, 4 h), followed by an OFX challenge (5 μg·mL^{-1}). After 6 h in the presence of OFX, the cells were perfused with fresh medium, allowing the persister cells to recover. The persister cell and its progeny cells during the OFX treatment and after OFX removal are highlighted in green and blue, respectively. The corresponding time points are indicated on each panel. Scale bar = 5 μm. Please click here to view a larger version of this figure.

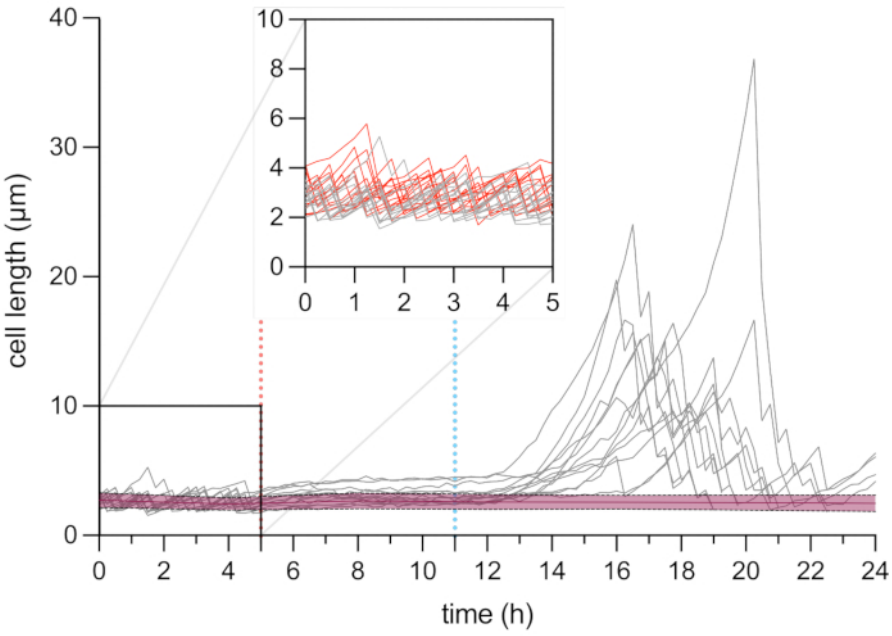

Figure 6: Microscopy time-lapse analysis of the length of the persister and dead cells. Cell length analysis of dead cells (in red, n = 109) and persister cells (in grey, n = 13). The start of the OFX treatment (5 µg·mL^{-1}) is indicated by the red dashed line (5 h), and the OFX removal is indicated by the blue dashed line. The inset corresponds to the growth phase before OFX addition. The experiments were performed in triplicate. The shades and dashed lines indicate the standard deviations for the dead cell population (n = 109). Please click here to view a larger version of this figure.

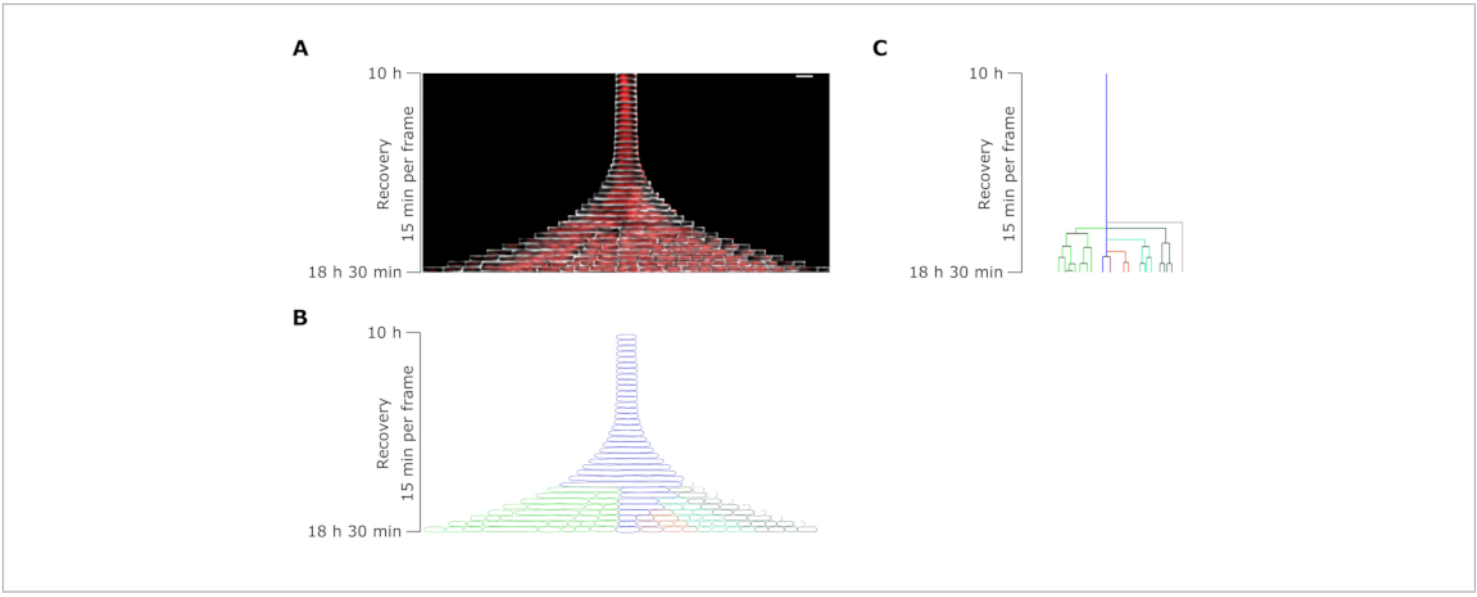

Figure 7: Microscopy time-lapse analysis of a representative persister to OFX. (**A**) Kymograph of a representative OFX persister and its daughter cells generated by filament divisions during 8.5 h after OFX removal (18.5 h after the beginning of the microfluidic experiment, comprising 4 h of growth, 6 h of 5 µg·mL^{-1} OFX treatment, and 8.5 h of recovery after OFX removal). One frame corresponds to 15 min. Scale bar = 5 µm. (**B**) Mask generated from the persister kymograph in **A**. The monitored persister cell is indicated with a blue outline, and the daughter cells are highlighted in distinct colors. (**C**) Schematic representation of the persister cell lineage generated from **B**. The color coding is identical to **B**. Please click here to view a larger version of this figure.

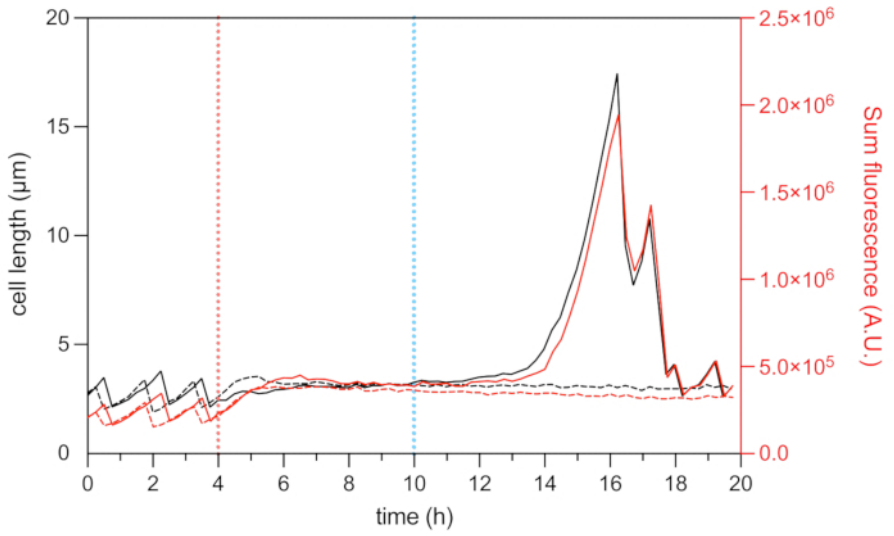

Figure 8: Cell length and mCherry fluorescence analysis of representative persister and dead cells. Analysis of the cell length (left axis) and the total HU-mCherry fluorescence intensity (right axis, shown in arbitrary units) of a representative persister (solid black and red lines) and a representative dead cell (dashed black and red line) during the microfluidic time-lapse experiment. The start of the OFX treatment (5 µg·mL^{-1}) is indicated by the red dashed line, and the OFX removal by the blue dashed line. Please click here to view a larger version of this figure.

Video 1: Microfluidic video of *E. coli* HU-mCherry cells before, during, and after ofloxacin treatment. Microfluidic time-lapse imaging showing HU-mCherry cells. The cells were grown for 4 h in MOPS glycerol 0.4%. After 6 h of OFX treatment (5 µg·mL^{-1}), the antibiotic-free medium was perfused in the microfluidic plate to allow the persister cells to recover. Scale bar = 5 µm. Time (in min) is indicated. The growth and recovery phases are indicated by "MOPS- Gly. 0.4%" and the OFX treatment by "OFX 5 µg/mL". Please click here to download this Video.

10x MOPS			
	Stock solution	**Volume of stock solution for 1 L of 10x MOPS**	**Final concentration in 10x MOPS base**
MOPS acid	1 M (adjusted to pH 7.4 using KOH)	400 mL	0.4 M
Tricine	1 M (adjusted to pH 7.4 using KOH)	40 mL	0.04 M
$FeSO4.7H_2O$	0.01 M	10 mL	0.0001 M

NH_4Cl	1.9 M	50 mL	0.095 M
K_2SO_4	0.276 M	10 mL	0.00276 M
$CaCl_2.2H_2O$	0.0005 M	10 mL	0.000005 M
$MgCl_2.6H2O$	0.528 M	10 mL	0.00528 M
NaCl	add directly 29.2 g		0.5 M
Distilled water		460 mL	
Micronutriments 1000x (see Table 2)		10 mL	

Table 1: Composition of 10x MOPS.

Micronutriments 1000x		
	Concentration in Micronutriments 1000x stock solution	**Final concentration in 10x MOPS base**
$(NH4)6Mo7O24.4H_2O$	0.000003 M	0.00000003 M
H_3BO_3	0.0004 M	0.000004 M
$CoCl_2.6H_2O$	0.00003 M	0.0000003 M
$CuSO_4.5H_2O$	0.00001 M	0.0000001 M
$MnCl_2.4H_2O$	0.00008 M	0.0000008 M
$ZnSO.7H_2O$	0.00001 M	0.0000001M

Table 2: Composition of 1,000x micronutrients.

MOPS glucose 0.4% or MOPS glycerol 0.4%			
	Stock solution	**Volume for 1 L MOPS glucose 0.4 % or MOPS glycerol 0.4 %**	**Final concentration in MOPS glucose 0.4% or MOPS glycerl 0.4%**
10x MOPS	see Table 1	100 mL	
K_2HPO_4	0.132 M	10 mL	0.00132 M
Glucose (for MOPS glucose 0.4%)	20% (20 g in 100 mL distilled water)	20 mL	0.40%
Glycerol (for MOPS glycerol 0.4%)	≤99%	4 mL	0.40%
Distilled water		870 mL for MOPS glucose 0.4% or 886 mL for MOPS glycerol 0.4%	

Table 3: Composition of MOPS glucose 0.4% and MOPS glycerol 0.4%.

Discussion

The protocol presented in this paper allows the analysis of the persistence phenotype observed in response to antibiotic treatment at the population and single-cell levels. The experiments were performed with the *E. coli* MG1655 strain, which was grown in a chemically defined medium (MOPS glycerol 0.4%). Time-kill assays and microscopy experiments were carried out on exponential-phase cultures. We used OFX, a fluoroquinolone, at a concentration of 5 $\mu g \cdot mL^{-1}$ to reveal the persister cells. The approaches described here can be applied to other bactericidal antibiotics, such as β-lactams, aminoglycosides, or antimicrobial compounds[31]. Accordingly, other bacterial strains, media, or growth conditions can be used. Monitoring different fluorescent fusions in a similar setup to that described here can be useful for following cellular processes such as DNA replication[32], DNA repair[25,33], and cell division[34] before, during, and after the antibiotic treatment. Similarly, fluorescent reporters can be exploited to investigate distinct aspects of cell physiology, such as intracellular pH[35], ATP[36], or ROS[37] levels. Alternatively to fluorescent fusions, chemical dyes could also be applied. For example, the *hupA-mCherry* fusion could be replaced by 4',6-diamidino-2-phenylindole (DAPI), a fluorescent dye that stains DNA[38]. Performing time-lapse microscopy coupled with such fluorescent dyes should, however, be avoided, as these staining techniques can perturb the dynamics of the cell cycle during time-lapse experiments. Alternatively, such experiments can be replaced by time-course analyses of snap-shot imaging at relevant timepoints.

While such fluorescent reporters are helpful, the amount of information that can be extracted through the analysis of phase-contrast images should not be neglected. Here, we monitored cell length evolution throughout the growth, OFX

treatment, and recovery stages. Other parameters based on phase-contrast images, such as cell width, phase-contrast intensity, and curvatures of the bacterial cells, can also be extracted with ease by using adequate software, such as MicrobeJ[27].

In summary, the procedure described here can be applied to other conditions and bacterial species to monitor cellular responses to changing environments or stressors[18,19]. By using other fluorescent reporters (transcriptional and translational reporters, chemical dye) in combination with a population analysis, such as flow cytometry/FACS, interesting questions can be addressed in a multi-scale framework.

Disclosures

The authors declare no competing interests.

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