Experimental Evolution of *Escherichia coli* Persister Levels Using Cyclic Antibiotic Treatments

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Abstract

Persister cells are difficult to study owing to their transient nature and their usually small number in bacterial populations. In the past, numerous attempts have been made to elucidate persistence mechanisms. However, because of the challenges involved in studying persisters and the clear redundancy in mechanisms underlying their generation, our knowledge of molecular pathways to persistence remains incomplete. Here, we describe how to use experimental evolution with cyclic antibiotic treatments to generate mutants with an increased persister level in stationary phase, ranging from the initial ancestral level up to 100 %. This method will help to unravel molecular pathways to persistence, and opens up a myriad of new possibilities in persister research, such as the convenient study of nearly pure persister cultures and the possibility to investigate the role of time and environmental aspects in the evolution of persistence.

Keywords: Experimental evolution, Persister mutants, Adaptation, Cyclic treatments, *Escherichia coli*, Bet-hedging, Aminoglycosides

1 Introduction

Persistence is a bet-hedging strategy that allows microbial populations to avoid eradication by otherwise lethal antibiotic treatments through the formation of antibiotic tolerant variants (usually 0.0001-0.1 % of the total population) as a first line of defense [1, 2]. Persisters do not divide during antibiotic treatment and can only resume growth after reverting to the antibiotic-sensitive non-persister phenotype when the antibiotic concentration has dropped, distinguishing them from resistant cells [3]. As such they can avoid clinical detection while causing therapy failure and the resurgence of infection, especially given their presence in biofilms where they are shielded from immune components [4].

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Several groups have searched for persistence genes and mechanisms using classical approaches like screening mutant libraries [5–13], overexpression libraries [14, 15], and transcriptome studies on isolated populations enriched for persisters [16–19]. Although progress has been made and several genes and mechanisms have been reported recently [20, 21], many questions remain [22]. The usefulness of genetic screens is inherently limited for the study of complex traits such as persistence, and the rare and transient character of the persister phenotype makes transcriptomic analysis impossible without cumbersome enrichment protocols.

We propose experimental evolution as an alternative approach to study persistence for the following reasons. First, experimental evolution is a powerful tool for studying complex, redundant phenotypes [23]. Second, there is no bias in the type of mutations that can be found (e.g. gain-of-function mutations and mutations in regulatory domains or essential genes) [24]. Third, it has already been shown that persister levels are evolvable, both in vivo [25, 26] and in vitro [27]. Here, we demonstrate a method to evolve *Escherichia coli* populations and generate mutants producing a range of persister levels up to 100 % in stationary phase.

2 Materials

Prepare all solutions and media using deionized water and store at room temperature unless stated otherwise. Use *E. coli* strains carrying unique markers as ancestor. For example, we use *E. coli* BW25993 carrying different fluorescent markers [28] (see **Note 1**). Always follow safety instructions when handling hazardous goods or equipment. When disposing waste, please follow local regulations and make sure biological waste is sterilized properly in advance.

2.1 General Material

1. Antibiotic stock solution: amikacin 50 mg/ml (see **Note 2**). Weigh 50 mg of amikacin powder stored at 4 °C and add sterile ultrapure water (resistivity of 18.2 MΩ/cm at 25 °C) to a final volume of 1 ml. Filter sterilize (0.22 μm) and store aliquots immediately at –20 °C (see **Note 3**).
2. Mueller Hinton Broth (MHB): follow the instructions of the supplier of premixed MHB powder (see **Note 4**).
3. Lysogeny Broth (LB) agar: weigh 10 g tryptone, 10 g NaCl, 5 g yeast extract and 15 g agar and add water to a final volume of 1 l before autoclaving. Keep the sterile broth on at 50–60 °C for maximum 2 days before pouring. Store plates at 4 °C for up to 1 month before use.
4. Magnesium sulfate solution (MgSO₄, 10 mM): weigh 2.46 g of MgSO₄·7H₂O and add water to a final volume of 1 l before autoclaving.

5. Sterile glass test tubes and Erlenmeyer flasks of 250 ml (*see Note 5*).
6. Sterile plastic tubes of 15 and 50 ml, suitable for centrifugation.
7. Microcentrifuge capable of $4000 \times g$.
8. Centrifuge for 50 ml tubes capable of $4000 \times g$.
9. Incubator at 37°C , fitted with trays for test tubes, microplates and Erlenmeyer flasks, capable of shaking at 200 rpm (*see Note 6*).
10. Spiral plater and automated colony counter or alternatively, sterile glass beads for manual plating and counting.

2.2 Determination of Antibiotic-Related Characteristics of Ancestral Strain

1. MHB agar plates containing different antibiotic concentrations: add 15 g of agar to 1 l of MHB before autoclaving. Keep the sterile broth on at $50\text{--}60^\circ\text{C}$ for maximum 2 days before pouring. Mix the sterile broth with amounts of antibiotic stock solution to reach the desired concentrations before pouring. Store MHB plates containing antibiotics at 4°C for up to 1 week before use [29].
2. Sterile 96-well microplate with lid and breathable sterile adhesive seals.
3. 96-well OD_{595nm} reader.

2.3 Evolution Experiment

1. Micropipette tips with filters.
2. Disposable gloves.
3. Disinfection ethanol 70 % (v/v): mix 30 parts of water with 70 parts of denatured ethanol.
4. Cryoprotectant: glycerol 50 % (v/v): mix 50 parts of water with 50 parts of glycerol.
5. Polypropylene tubes suitable for -80°C preservation (cryotube).
6. Fluorescence microscope (*see Note 7*).

3 Methods

Perform all handlings at room temperature and work under sterile conditions. Incubation is carried out at 37°C , shaking at 200 rpm for liquid cultures. Centrifugation steps are performed at $4000 \times g$.

3.1 Characterization of Antibiotic-Related Characteristics of Ancestral Strain

The minimum inhibitory concentration (MIC), the minimum bactericidal concentration (MBC), the mutant prevention concentration (MPC) and the antibiotic concentration and treatment duration needed to reach the persister plateau must be determined for the ancestor before the start of the evolution experiment, and for evolved populations and/or clones afterwards (*see Notes 8 and 9*). Subheadings 3.1.1 and 3.1.2 are adaptations from previously described methods [30–32].

3.1.1 MIC and MBC Determination

1. Inoculate the ancestral strain (*see* **Notes 1** and **9**) in a test tube containing 5 ml of MHB and incubate overnight.
2. Adjust the optical density of the overnight culture at 595 nm (OD_{595nm}) to 0.5 by centrifugation in a microcentrifuge (5 min) and resuspending and diluting in MHB.
3. Dilute the OD_{595nm} -adjusted suspension 1/100 in 40 ml MHB and mix well. Add antibiotic to 10 ml of this inoculum to a final concentration of two times the highest concentration that you want to assess (*see* **Note 10**).
4. Add 150 μ l of the remaining 30 ml of bacterial inoculum from **step 3** to the wells of a sterile microplate. Use three rows as technical replicates per antibiotic per clone and leave column 12 blank. Make also an appropriate tenfold serial dilution series in $MgSO_4$ solution (10 mM) of the remaining inoculum and plate out on LB agar. Count colonies after overnight incubation to determine the CFU per ml.
5. Add 150 μ l of MHB to column 12 as negative control.
6. Make a twofold serial dilution series of the antibiotic: add 150 μ l of the inoculum with antibiotic to the wells of column 1, mix, and transfer 150 μ l to the next column and repeat for each column until column 10. Mix column 10 and remove 150 μ l. Leave column 11 without antibiotics as a positive control. Cover the microplate with a breathable seal and plastic lid, and incubate for 16–20 h.
7. Measure the OD_{595nm} for each well in a multireader. Verify the positive and negative controls for adequate microbial growth and medium sterility. The MIC is the lowest concentration inhibiting visible growth.
8. Make appropriate tenfold serial dilutions in $MgSO_4$ (10 mM) of the wells containing antibiotic above the MIC (thus showing no visible growth) and plate out on LB agar. Count colonies after overnight incubation to determine the CFU per ml and compare with CFU per ml of the inoculum determined in **step 4**. The MBC is the lowest concentration capable of killing 99.9 % of cells.

3.1.2 MPC Determination

1. Inoculate the ancestral strain (*see* **Notes 1** and **9**) in a test tube containing 5 ml of MHB and incubate overnight.
2. Dilute the overnight culture 1/100 in a flask containing 100 ml of MHB and incubate overnight.
3. Concentrate the overnight culture tenfold: centrifuge 50 ml of the overnight culture (25 min), discard the supernatant and resuspend the cell pellet in 5 ml of $MgSO_4$ solution (10 mM).
4. Plate out $>10^{10}$ cells on MHB plates containing a twofold serial dilution range of antibiotic (*see* **Note 10**). We use five

plates per antibiotic concentration and plate out 200 μ l of the cell suspension prepared in **step 3** on each plate. Incubate for 48 h to allow detection of colonies arising from resistant mutants.

5. The MPC is the lowest concentration inhibiting growth of resistant mutants.

3.1.3 Construction of Killing Curves in Function of Antibiotic Concentration and Treatment Duration to Determine the Persister Plateau

1. Inoculate the ancestral strain (*see* **Notes 1 and 9**) in a test tube containing 5 ml of MHB and incubate overnight.
2. Dilute the overnight culture 1/100 in a flask containing 100 ml of MHB and incubate overnight.
3. Transfer samples of 1 ml of the overnight culture to test tubes and incubate in the presence of a range of antibiotic concentrations for 5 h. Alternatively, samples can be treated with a fixed antibiotic concentration for various treatment durations to determine killing curves in function of time (*see* **Note 10**).
4. Make an appropriate tenfold serial dilution series of the overnight culture in MgSO_4 solution (10 mM) and plate out on LB agar. Count colonies after overnight incubation to determine the initial cell number.
5. After antibiotic treatment, spin the samples down in a microcentrifuge (5 min), wash once with MgSO_4 solution (10 mM) to remove the antibiotic, make appropriate tenfold serial dilutions in MgSO_4 solution (10 mM) and plate out on LB agar plates. Count colonies after 48 h of incubation to determine the number of surviving cells (*see* **Note 11**).
6. Plot the number of surviving cells in function of antibiotic concentration or treatment duration to determine the conditions needed to reach the persister plateau (*see* **Note 8**) (Fig. 1).

3.2 Evolution Experiment

Since evolution experiments often require many days of continuous lab work and many factors can confound the outcome of the experiment (e.g. contaminations, timing of growth and treatment periods, accidental events like power breakdown or glassware breakage and chance resulting in genetic drift), we want to stress the importance of material preparations, thoughtful experimental set-up and careful handling of evolving populations (*see* **Notes 3–5, 7, 9 and 12–19**) to improve replicability and increase work efficiency.

To start the evolution experiment:

1. Inoculate separate single colonies in test tubes containing 5 ml of MHB and incubate overnight. One colony is used as the founder of each population (*see* **Notes 12 and 13**).
2. Dilute the overnight cultures 1/100 in flasks containing 100 ml of MHB and incubate overnight (*see* **Note 14**).

Fig. 1 Killing curve in function of treatment duration or antibiotic concentration reveals a persister plateau. Initially, the number of surviving cells decreases rapidly with increasing antibiotic concentration or treatment duration. However, at a certain point a plateau is reached where a further increase in concentration or treatment duration only slightly increases killing. At this point, all normal cells are killed, and only highly tolerant persister cells remain. This biphasic pattern is a result of the initial fast killing of normal cells (*gray*) and the increased survival of persisters (*black*) at longer treatment times or higher antibiotic concentrations. Antibiotic concentration and treatment time should be chosen to fall within the range sufficient to reach the persister plateau and ideally in a range where the surviving fraction is minimally influenced by small experimental fluctuations. Reproduced and adapted with permission from [20]

3. Depending on the desired treatment frequency, days with treatment should be alternated with days without treatment (*see Note 15*). If the overnight culture (resulting from **steps 2, 6 or 8**) needs to be treated, continue with **step 4**. If not, move on to **step 8**. In case you want to end the experiment, proceed to **step 9** (*see Note 16*).

On days with treatment:

4. Make appropriate tenfold serial dilutions of the overnight cultures in MgSO₄ (10 mM), plate out on LB agar and incubate overnight to determine the total CFU per ml.
5. Treat a 2 ml sample (2 × 1 ml) of each overnight culture with antibiotic. Use a concentration and treatment duration according to the results obtained in Subheading 3.1 (*see Note 8*).
6. After antibiotic treatment, spin down the samples in a microcentrifuge (5 min) and wash three times with MgSO₄ solution (10 mM) to remove the antibiotic. Dilute each sample 1/100 in flasks containing 100 ml of MHB and incubate overnight to

start another cycle of growth (*see* **Note 14**). Make appropriate tenfold serial dilutions of the remaining of the washed samples in MgSO_4 solution (10 mM), plate out on LB agar and incubate for 48 h to determine CFU per ml of surviving cells (*see* **Note 11**). The ratio of CFU per ml of surviving cells to CFU per ml of total cells is defined as the persister fraction and should be monitored at regular time intervals throughout the experiment.

7. Irrespective of the desired treatment frequency, pellet 50 ml of the overnight cultures resulting from **step 6** by centrifugation (25 min). Resuspend the pellet in MgSO_4 solution (10 mM) to a final volume of 1 ml, add 1 ml of glycerol (50 %, v/v) and store immediately at -80°C (*see* **Note 16**). Go to **step 3**.

On days without treatment:

8. Dilute the overnight cultures from **step 6** or **step 8** 1/1,000,000 in flasks containing 100 ml of MHB and incubate overnight to start another cycle of growth (*see* **Notes 14** and **17**). Go to **step 3**.

To end or interrupt the experiment (*see* **Note 16**):

9. Check every population for contaminations. We use different fluorescent markers to check for possible contamination using fluorescence microscopy (*see* **Notes 1** and **18**).
10. Cryopreserve the overnight cultures as described in **step 7**.
11. Plate out the overnight cultures as described in **step 4**.
12. Isolate clonal endpoints: pick single colonies and use these to inoculate test tubes containing 5 ml of MHB (*see* **Note 19**). Incubate overnight.
13. Cryopreserve the clones at -80°C : mix 1 ml of overnight culture with 1 ml of glycerol (50 %, v/v).
14. Analyze the antibiotic-related characteristics of the clones and/or the populations according to Subheading 3.1 (*see* **Notes 8** and **9**).

4 Notes

1. We have used numerous *E. coli* lab strains with different genetic backgrounds but also pathogenic strains and even other species (e.g. *Salmonella* Typhimurium) to evolve high persister levels. Ideally, the selected strain should allow detection of (cross)-contamination (e.g. different fluorescent markers, auxotrophic markers), especially in initial experiments. Any extra information (e.g. genomic sequence) can also be taken into account when choosing an ancestral strain. Evidently, the strain should be sensitive to the used antibiotic.

2. We have confirmed our method with other aminoglycosides (kanamycin, tobramycin, and gentamicin). The concentration of the stock solution may be adapted in function of the desired final concentration. When shifting from formulation (e.g. hydrate, sulfates) or supplier, take into account the possible potency difference to achieve similar concentrations.
3. When using antibiotic stock solutions over a long time period (e.g. during a long-term evolution experiment), minimize freeze–thaw cycles of aliquots and renew antibiotic stocks frequently to avoid attenuation of antibiotic efficacy. Amikacin should not be stored for longer than 1 month at $-20\text{ }^{\circ}\text{C}$ [29]. Therefore, adjust the amount of antibiotic stock solution and volume of the aliquots to the expected needs.
4. Using premixed MHB formulas may enhance reproducibility. Since company formulas may differ, it is recommended to stick to one supplier and manage MHB stocks well to tackle accidental events in advance (e.g. problems with deliveries or differences between batches). Alternatively, MHB can be prepared using 2 g/l beef Extract powder, 17.5 g/l acid digest of casein, and 1.5 g/l starch. In some cases (depending on the organism and antibiotic of interest [30]) Ca^{2+} and Mg^{2+} content of MHB needs to be adjusted to final concentrations of 20–25 mg/l and 10–12.5 mg/l, respectively. Transfer MHB to other recipients (test tubes, Erlenmeyer flasks) before autoclaving and under continuous mixing or after gentle heating to dissolve it completely. When preparing MHB medium for the evolution experiment itself, please reserve sufficient MHB powder of the same batch for the entire experiment, never store the medium after autoclaving for longer than a week, store at room temperature and away from light and never autoclave twice.
5. Make sure to provide at least three times the number of populations in Erlenmeyer flasks when starting an evolution experiment. Depending on the turnover of your institution's glassware cleaning and the length of the experiment, four or five times the number of populations in flasks may be required.
6. For comparison between parallel evolutionary lines and to compare between independent phenotyping experiments, it is best stick to one incubator or use incubators with identical properties (e.g. shaking amplitude). In our case, 200 rpm equals to 0.11 g.
7. Depending on the unique markers present in the ancestral *E. coli* strains, other means may be needed to check for contamination.
8. These parameters need to be determined at least in biological triplicate. We recommend the antibiotic concentration in the evolution experiment to be at least ten times as high as the MIC

and MBC values, and well above the MPC. The antibiotic concentration and treatment duration also need to be sufficiently high to reach the persister plateau. In our case, antibiotic treatments using amikacin concentrations of minimum 50 µg/ml and maximum 400 µg/ml for 5 h were successfully used in the evolutionary set-up. After the evolution experiment, Subheading 3.1 can be used to assess evolution of genuine persistence, i.e. increased survival with no accompanying changes in MIC value, and to assess evolution of the other antibiotic-related characteristics.

9. If using evolved populations, it is best to limit the amount of growth cycles to reduce further evolution and change in population compositions. Therefore, for populations, Subheading 3.1 could be performed during the evolution experiment. Alternatively and more practically, frozen stocks can also be used as starting point. We advise to thaw these stocks completely and dilute them 1/100 in MHB medium to allow for one overnight growth cycle to stationary phase to obtain sufficient starting material for Subheading 3.1.
10. MIC, MBC, MPC and concentration and treatment durations needed to reach a persister plateau can vary between different species and even between *E. coli* strains. If no prior information is available, we advise starting with a broad range and/or few concentrations. In order to determine the concentrations and treatment durations needed to reach a persister plateau, we always start with optimizing the antibiotic concentration using a fixed and practical treatment time (e.g. 5 h, which leaves enough time to complete growth to stationary phase during a total cycle of 24 h). Subsequently, the persister plateau should be verified in function of time.
11. Since persister cells have longer lag times than normal cells [2, 27], we incubate for 48 h instead of 24 h when plating out after treatment to ensure colony formation of most persister cells.
12. Founding each population from a different colony can prevent compromised results due to the influence of random mutations that might be present in some initial clones. We also advise to replica plate these colonies at the start of the experiment on LB agar and cryopreserve part of the first overnight cultures from **step 1** at -80°C , e.g. by mixing 1 ml of culture with 1 ml of glycerol (50 %, v/v) to be able to track back particular observations to the founding colony. Depending on the objective of the experiment, the number of replicate populations can be increased or decreased. For example, for a try-out experiment or when the goal is to obtain a high persister mutant for practical reasons in a follow-up study, 1–3 replicates might suffice.

In other cases, for example when the goal is to follow evolution over longer timescales or to study the dynamics of the evolutionary process, it is advised to increase the number of replicate populations to reduce the effect of chance and increase the study's resolution. However, keep in mind that each increase in number of replicates also implies increased work load, materials, and infrastructure demands. If high numbers of replicates are nevertheless necessary, good preparation becomes especially essential (*see* Subheading 2).

13. If you have access to isogenic tagged strains (*see* **Note 1**), we encourage to use them as founders for each population, especially in initial experiments. If you have more populations than uniquely marked strains, we suggest to alternately handle populations with contrasting markers. In this way, cross-contamination may be identified more easily. To limit contamination of any kind, we advise to wear gloves, disinfect gloves and materials between handling different populations, use micropipette tips with filters when handling evolving populations and exclusively use freshly autoclaved solutions of glycerol (50 %, v/v) and MgSO_4 solution (10 mM). Never open two flasks or recipients containing evolving populations at the same time.
14. To avoid accidental events that might result in the premature ending of the experiment, we advise storing stationary phase cultures on days with and without treatment at 4 °C as backup until populations have reached the next stationary phase.
15. Although treatment frequency is generally expressed as “once every x days”, identical treatment frequencies can be obtained in different ways. For example, when the treatment frequency is once every 8 days, the set-up can start with a treatment followed by 7 dilution steps, start with 7 dilution steps and end with a treatment, or any situation in between. For uniformity, we always start with a treatment step followed by dilution steps in any given setup. On days without treatment, persister levels can still be assayed if necessary, analogous to **steps 4–6** but without inoculating the next cycle of evolution with persister cells.
16. At the last day of the evolution experiment, we once again assay persister levels by repeating **steps 4–6** and freeze the resulting overnight culture like in **step 7**. In this way, the evolution experiment can always be restarted if necessary. Although we advise to perform the experiment without interruption, this allows to pause the experiment if needed. It is possible and sometimes advisable to store samples at –80 °C more frequently as backup and cryopreserved evolution library.

As a minimum, we advise to cryopreserve overnight cultures after every treatment cycle. In this way, if you need to start over, the populations can be inoculated from completely thawed cryopreserved backups with a dilution factor similar to **step 8**. Furthermore, the populations then have at least one growth cycle to adjust from -80°C conditions before treatment starts. In case the treatment frequency is once every day, persister cells at the end of the experiment (*see Note 14*), or at intermediate points, can be cryopreserved at -80°C after removal of the antibiotic as in **step 6**.

17. On days without treatment, we dilute samples $1/1,000,000$ to mimic the bottleneck populations experience on treatment days. Depending on the initial persister level (in our case 10^{-3} - 10^{-4}), we advise to adjust this dilution factor. However, keep in mind that strong bottlenecks increase the effect of genetic drift, especially when initial persister levels are low. Furthermore, when using lower treatment frequencies, the selection pressure is not as strong, increasing the influence of drift [33].
18. We advise to check for contamination also at intermediate points during the experiment and definitely when storing samples to pause the experiment. Since the presence of a small number of non-fluorescent cells within a whole population might be difficult to observe, repeat the contamination check on the randomly selected clones.
19. The number of clonal endpoints to isolate from each population is somewhat arbitrary, but again depends on the goal of the experiment. If treatment frequency is low, we would recommend to pick at least five clones per population since variability within populations and between replicate populations might be high. If treatment frequency is high, the number of clones (and the number of replicate populations to isolate clones from) may be decreased.

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