

Chapter ? - The use of experimental evolution to study the response of *Pseudomonas aeruginosa* to single or double antibiotic treatment

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The use of experimental evolution to study the response of *Pseudomonas aeruginosa* to single or double antibiotic treatment

Summary

The widespread use of antibiotics promotes the evolution and dissemination of drug resistance and tolerance. Both mechanisms promote survival during antibiotic exposure and their role and development can be studied *in vitro* with different assays to document the gradual adaptation through the selective enrichment of resistant or tolerant mutant variants. Here, we describe the use of experimental evolution in combination with time-resolved genome analysis as a powerful tool to study the interaction of antibiotic tolerance and resistance in the human pathogen *Pseudomonas aeruginosa*. This method guides the identification of components involved in alleviating antibiotic stress and helps to unravel specific molecular pathways leading to drug tolerance or resistance. We discuss the influence of single or double drug treatment regimens and environmental aspects on the evolution of antibiotic resilience mechanisms.

1. Introduction

The great therapeutic achievements of antibiotics for human health have been dramatically undercut by the steady evolution of survival strategies allowing bacteria to overcome antibiotic action (1, 2). While resistance plays a major role in antibiotic-treatment failure, bacteria can use other mechanisms such as tolerance to survive antibiotic treatment (3). Resistance is generally drug specific and leads to an increase of the minimum inhibitory concentration (MIC). Tolerance is the ability of antibiotic-sensitive bacteria to survive during transient exposure to different antibiotics at concentrations that exceed their MIC for a particular drug (4). Experimental evolution has proven an effective tool to probe the gradual accumulation of antibiotic resistance (5-9) and tolerance (10-15) as well as to study how drug tolerance can influence the development of drug resistance (16-19). Here, we introduce an experimental evolution protocol that implements recurrent population genome sequencing to investigate gradual genetic adaptations of evolving populations. This allows following the gradual evolution of sensitive ancestors of *P. aeruginosa* into different competing sub-lineages carrying single or multiple mutations conferring tolerance or resistance and its dependence on the specific drug treatment applied. By evolving multiple lineages in parallel, this approach provides a comprehensive view of the adaptation processes and of the genetic landscape behind these processes. This experimental evolution protocol uncovers important tolerance determinants and reveals the role of low-level resistance mutations in the promotion of *P. aeruginosa* survival at high therapeutic antibiotic doses. This is of particular importance, as it allows to dissect the specific contribution of *bona fide* tolerance mutations and resistance to the overall survival of this important human pathogen during specific pharmacokinetic regimens (20).

2. Materials

Prepare all solutions and media using deionized water and store at room temperature unless stated otherwise.

2.1. General materials

1. Antibiotic stock solution of 10 mg/mL tobramycin and 2 mg/mL ciprofloxacin :
Dissolve 100 mg of tobramycin powder stored at 4 °C in 10 mL of sterile ultrapure water or dissolve 5 mg of ciprofloxacin powder stored at 4 °C in 2.5 mL of sterile ultrapure water. Filter sterilize (0.22 µm) and store aliquots immediately at -20 °C.
(see **Note 1**).
2. Lysogeny Broth (LB) medium : Dissolve 10 g tryptone, 5 g yeast extract and 10 g NaCl in 1 L deionized water, and autoclave for 30 min at 121 °C (see **Note 2**).
3. LB agar : Weigh 10 g tryptone, 5 g yeast extract, 10 g NaCl and 15 g agar and add water to a final volume of 1 L before autoclaving. Allow the medium to cool to 50-60 °C before pouring in Petri dishes. Store plates at 4 °C no longer than one month.
4. Sterile glass test tubes and Erlenmeyer flasks of 100 mL (see **Note 3**).
5. Sterile plastic tubes of 15 and 50 mL, suitable for centrifugation.
6. Spectrophotometer.
7. Benchtop microcentrifuge capable of 4,000 g.
8. Centrifuge for 50 mL tubes capable of 7,000 g.
9. PCR thermocycler.
10. Incubator at 37 °C capable of shaking at 170 rpm.
11. Multichannel pipette for plating.

2.2. *In vitro* evolution experiment

1. LB-DMSO cryoprotectant – LB medium with 15 % (v/v) DMSO : Mix 15 parts of DMSO with 75 parts of LB.
2. Polypropylene tubes suitable for -80 °C preservation (cryotubes).

2.3. Determination of MIC

1. Sterile 96-well microplates with lid and breathable sterile adhesive seals.
2. Plate reader (OD600 nm).

2.4. Genomic DNA preparation

1. GenEluteBacterial Genomic DNA kit (Sigma) or equivalent.
2. Nanodrop spectrophotometer.
3. 10x TBE buffer : Dissolve 108 g Tris base and 55 g boric acid in 900 mL of water and 40 mL of 0.5 M EDTA solution (pH 8.0). Adjust the volume to 1 L before autoclaving.
4. 0.6 % agar gel : Weigh 0.6 g of agarose, add 100 mL of TBE buffer before melting in the microwave.

2.5. Isolation of hyper-tolerant or drug resistant strains

1. Sterile 96-well microplates with lid and breathable sterile adhesive seals.

3. Methods

Perform all handlings at room temperature and work under sterile conditions. Incubation is carried out at 37 °C, shaking at 170 rpm for liquid cultures. Centrifugation steps are performed at 4,000 g in the microcentrifuge and at 7,000 g in the larger floor model centrifuge.

3.1. Evolution experiment

Evolution experiments often include multiple consecutive days of lab work. Also, many factors can potentially confound the outcome of the experiment, including contaminations, timing of growth and treatment periods, accidental events like power breakdown or glassware breakage. To improve the reproducibility of the experiments and increase work efficiency, careful material preparations, thoughtful experimental set-up and careful handling of evolving populations are of critical importance. It is also important to note that protocols and drug concentrations used were optimized for *P. aeruginosa* (see **Note 4**).

To start the evolution experiment:

1. Streak the ancestor strain from LB-DMSO stock (see **Note 4**) on a fresh LB plate just before the start of the evolution experiment.
2. Inoculate single colonies into individual test tubes containing 5 mL of LB and incubate overnight. One colony is used as the founder of each evolving lineage (see **Note 5**).
3. Measure the optical density of the overnight cultures at 600 nm (OD₆₀₀) and dilute to an initial OD₆₀₀ of 0.12 (corresponding to $\sim 10^8$ CFU/mL) in Erlenmeyer flasks containing 20 mL of LB. For this, centrifuge the corresponding volume of bacteria in a 1.5 mL Eppendorf tube, remove the supernatant and resuspend the pellet in 20 mL of LB. Before adding the antibiotic or combination of antibiotics (see **Notes 1, 6 and 7**), withdraw an aliquot of bacteria (200 μ l is usually sufficient) to determine the total CFU per mL at time zero (T₀).
4. Add antibiotic(s) and incubate the flasks on a shaker for the entire duration of the experiment (see **Note 8**).

5. To determine CFUs per mL at T₀, make serial ten-fold dilutions of the harvested 200 µl aliquot, spot droplets of 10 µl (see **Note 9**) on LB agar plates and incubate plates overnight (see **Note 10**).
6. Harvest an aliquot for the freezer stock. Centrifuge 1 mL of the overnight cultures resulting from step 2 for 1 min, resuspend the pellet in 1 mL of LB-DMSO and store immediately at -80 °C (see **Note 11**).
7. Harvest an aliquot for population genome sequence analysis (see **Note 11** and section 3.3). Centrifuge 1 mL of the overnight cultures resulting from step 2 for 1 min, remove the supernatant and store the cell pellet immediately at -80 °C.
8. Harvest an aliquot to determine the MIC (see section 3.2).
9. Harvest an aliquot to determine CFUs. Make appropriate serial ten-fold dilutions of the samples in LB medium, spot droplets on LB agar plates and incubate up to 48 h to quantify surviving cells (see **Notes 9, 10 and 12**). The fraction of survivors is calculated by dividing the number of CFU/mL after 3 h treatment by the number of CFU at T₀ (before antibiotic treatment).
10. Transfer the remaining culture to a 50 mL tube using a sterile pipette to minimize cross-contamination (see **Note 13**), centrifuge for 10 min and wash twice with LB to remove the antibiotic (see **Note 12**). Resuspend the bacterial pellet in a glass tube containing 5 mL of LB and incubate overnight to start the next growth cycle (Fig. 1) (see **Note 7**).
11. Go to step 3 and repeat the procedure with daily cycles until drug resistance or tolerance has evolved. Under these conditions, evolution of drug resistance is generally observed within 6-7 days for single drug treatment and low level resistance is observed within 8-9 days for double drugs treatment (see **Note 14**).

12. For data analysis, plot the fraction of survivors (step 9) and MIC values (see section 3.2) as a function of time. Include the fraction of cells carrying specific single-nucleotide polymorphisms (SNPs) as pie-chart for daily samples (see section 3.3). By directly linking the increase in resistance and bacterial survival with genetic changes occurring during evolution, these data allow discriminating mutations leading to resistance and tolerance. To establish causal relationships between genetic changes and antibiotic phenotypes, individual mutations need to be further characterization as describe in sections 3.4 and 3.5 (see **Note 15**).

3.2. MIC determination

MIC values are determined for the ancestor before the start of the evolution experiment and periodically for evolving populations and isolated clones during the experiment. Section 3.2 is an adaptation from previously described methods (*21*).

1. Dilute the overnight culture (step 3 of section 3.1) into LB medium to reach an OD₆₀₀ of 0.1.
2. Make two-fold serial dilutions of the antibiotic in a 96-well plate. To do so, add 200 μ L of LB to column 2 and supplement it with the antibiotic to reach the double of the highest concentration to be tested. Add 100 μ L of LB to columns 3-10 and transfer 100 μ l from each column to the next, starting with column 2. Add 100 μ L of LB to column 11 as a control lacking antibiotics. Add 100 μ L of the bacterial solution prepared in step 1 to each well and fill the wells at the edges with LB as a sterility control. Cover the microplate with a breathable seal and plastic lid, and incubate for 16-20 h.

3. Measure the OD₆₀₀ nm for each well in a microplate reader. Verify the positive and negative controls for adequate microbial growth and medium sterility. The MIC represents the lowest concentration with clear growth inhibition.

3.3. Preparation of gDNA and genome sequencing

Genomic DNA (gDNA) is extracted from the pellets of frozen overnight cultures (step 7 in section 3.1) of the evolution experiment.

1. Perform gDNA preparation following the manufacturer's instructions. We used the GenEluteBacterial Genomic DNA kit (Sigma) with exception of the final elution step, for which we used H₂O instead of the elution buffer provided with the kit.
2. Quantify the gDNA concentration with a Nanodrop spectrophotometer.
3. Monitor genomic DNA integrity by running an aliquot on a 0.6 % agar gel.
4. Analyze gDNA by Illumina sequencing. Generate the library and the sample barcoding with the NexteraXT approach (Illumina) and verify the library quality with a Fragment Analyzer (Advanced Analytical). Perform PE125 sequencing runs on parallel libraries on a HiSeq lane (Illumina) with a targeted coverage of about 100x.
5. Analyze DNA sequences. Map the sequencing reads onto the genome of the reference strain *P. aeruginosa* PAO1 (NC_002516) (see **Note 5**) with Bowtie2 (**22**) and small polymorphisms. Spot the structural and coverage variants with Samtools (**23**) and a collection of in-house Perl scripts (**24**) (see **Note 16**). Details for this analysis are provided in sections 3.3.1-3.3.3 below.

3.3.1. Alignment to a reference genome

In order to map the reads (see **Note 17**) onto a reference genome, we recommend to use the alignment freeware Bowtie2 that can be installed on Windows, MacOS or Linux OS

(<https://sourceforge.net/projects/bowtie-bio/files/bowtie2/>) (**22**). Bowtie2 is command line

software that requires the command terminal available on any given system. First, build index files from the desired reference. Together with the reads file (.fastq), the index files are used by Bowtie2 to perform the alignments against the target reference. This requires the sequence ‘.fasta’ file of the targeted chromosome (for example, *P. aeruginosa* PAO1 is NC_002516.2, available at NCBI).

```
> bowtie2-build reference.fasta reference.index
```

Reads from a given evolution time point (e.g. A) are mapped as follows:

```
> bowtie2 --local -a -R 3 -N 0 -L 20 -i S,1,0.50 -x ref.index -1  
A.PER.1.fastq.gz -2 A.PER.2.fastq.gz -S A.Assem.sam
```

The alignment is performed locally (--local, i.e. partial matches allowed), all matches are reported (-a), with high sensitivity parameters (-R 3 -N 0 -L 20 -i S,1,0.50). Here, two pair end read files are mapped (-1 PER.1.fastq.gz -2 PER.2.fastq.gz). If only a single read file is available, use -U Reads.fastq.gz instead (see **Note 18**).

```
# create a binary version of the assembly file  
  
> samtools view -F 0x0100 -b A.Assem.sam > A.Assem.bam  
  
# Order the file along the reference sequence
```

The resulting assembly file in ‘.sam’ or ‘.bam’ format (binary equivalent) contains the alignment information required for SNP calling and for the identification of recombination events for a single sample (i.e. evolution time point). In order to reformat the assembly file before the identification of possible mutations (i.e. variant calling), use the Samtools and BCFtools freeware that is available under Linux (<http://www.htslib.org/download/>) (23). If you do not have access to a Linux environment (e.g. <https://ubuntu.com/>), the software can be

emulated in MacOS or windows using a virtual machine solution (e.g.

<https://www.virtualbox.org/>).

```
#pileup file
```

Reference	base	coverage	match	match quality (ASCII coded)
AE004091.2_PAO1 3	T	43	^...^^.....^...^.....	GCG0EGGG0GGF1GGGE>GF1G0@GECCGFEGGEGCGGEG0GC
AE004091.2_PAO1 4	A	43	F0G0GGGG<GGB:GGECGF=G00FGGGCGGGGGGGGG0GG
AE004091.2_PAO1 5	A	43,A,,,,,a.....	G1G?GGGGFGGF1GGGGEG:@G0DG>GGGDGCGGGGEGGGCGG
AE004091.2_PAO1 6	A	43	aaaaAAaAaAaAAaaaaAA	GEGFGGGG@GGC1GGGGFGDCD9CGEFGGGEDGEFCFGGGFGE
AE004091.2_PAO1 7	G	43	GDC1FGGGFGGFDGGFGGG@G>00GG0FGGECGGEDGGGECGG
AE004091.2_PAO1 8	A	43	GCCF>GGGFGGFGDGGGGBGG<<G>DCGGGBGGCGGGG/GG

The ‘.pileup’ format of these output files (above) is convenient for script parsing as it summarizes all the information available for each chromosomal position in one line. It will be useful later for the manual analysis of the genetic variations spotted. The mpileup options are set in order to minimize data loss during possible recombination events occurring during the evolution experiment (-A -B -x -d 1000). Each read/reference match is reported in the 4th column of the pileup file (above):

- ‘.’ and ‘,’ indicate reference matches in the two DNA strands
- ‘ATGC’ and ‘atgc’ indicate substitutions
- insertion sequences are preceded by a ‘+’ or a ‘-’ sign followed by the length of the insertion or deletion (indel) and its sequence
- ‘\$’ and ‘^’ indicate ends of read alignments.

3.3.2. Call, selection and quantification of emerging alleles

Perform the variant calling as follows:

```
# Compile the assembly in BCFtool compliant format (-g)

samtools mpileup -g -A -B -x -d 1000 -f -f ref.fasta
A.Assem.sort.bam > A.Assem.sort.pileup.bcf

# Index the file prior base call
```

Only potential variant sites are reported (-v) in a parsable and human readable format (-O v) in the ‘.VarBase.vcf’ file. Before proceeding, all previous steps need to be executed (Bowtie2, Samtools & bcftools) with each sequenced sample (i.e time point).

For the final steps of the analysis, use the freely accessible Perl scripts to gather and refine the data generated up to this point (<https://github.com/Pablo-Manfredi/Experimental-Evolution-NGS>). Perl is a high-level and general-purpose scripting language with freely available interpreters (e.g. www.activestate.com) (24). The scripts can be pasted and modified on any notepad program and are listed with a .pl extension to be automatically recognized by the Perl interpreter. The scripts will automatically look for files present in the same folder. To have them working properly, copy the scripts in the folder containing the files to analyzed and keep the file extension system as recommended here. Feel free to modify the “A” prefix of the file name. For example, ‘Sample1.Assem.sam’, ‘Genome22.VarBase.vcf’, or ‘Day.3.Assem.sort.pileup.bcf.’

Once a variant call file (.vcf) per evolution time point is generated, copy the ‘ExtractList.pl’ Perl script in the directory containing the ‘.vcf’ files and execute it to reference all the potentially varying chromosomal positions in the course of the experimental evolution. The

file ‘SitesOfInterest.tsv’ generated by the script lists all chromosomal positions where a significant variant has been observed in at least one time point. The information can then be extracted from the ‘.sam’ assembly files using the ‘bcftools call’ again. However, this time positional information will also be extracted about sites where mutations did not occur yet in the sample. This is important in order to compare a variant position in sample A against a no variant position in sample B. Skipping this step would lead to the comparison of a variant position in sample A with the absence of information about the variant position in sample B. This might be erroneous as the absence of variant detection does not necessarily correspond to a reference allele (e.g. missing sequencing information).

```
# Perform base calling again on specific positions for each sample
bcftools call --regions-file SitesOfInterest.tsv -A -m --ploidy 1
```

This step needs to be repeated for all samples. Once all assemblies are screened for all positions varying along the experimental evolution, run the ‘TABmaker.SNP.pl’ Perl script in order to gather and filter all relevant information together in a ‘Evolution.tsv’ file that can be inspected with Excel-like programs. Proceed as you did previously with the ‘ExtractList.pl’ script. The ‘Evolution.tsv’ file describes a potentially varying position per line:

Contig	Posi	R/day1	R/day5	R/day11	R/day13	day1.R	day5.R	day11.R	day13.R
AE004091.2_PA01	1467483	NO	CG/CCGG	NO	CG/CGG	0	0.3	0	0
AE004091.2_PA01	2239555	AC/AGC	A/AG	A/AG	A/AG	0.9	0.8	0.9	0.9

- a. The first ‘R/sample’ block describes the different alleles observed at different time points (Reference/sample). ‘No’ only applies to insertion/deletion (indel) events and indicates that no indel event has been observed at this position.
- b. The second ‘sample.R’ block indicates the population ratios observed for the corresponding alleles. For example, 0.9 means that 90 % of the reads mapping on this

position carry the corresponding allele, while 10 % carry a ‘reference’ allele. 0 means no alternative allele has been detected at a given time point.

The setting of the ‘TABmaker.SNP.pl’ script allows introducing some false positive events (for example, in the case of small indels). Candidate positions need to be checked manually in each sample by using a simple Linux ‘sed’ command to extract information from the ‘.pileup’ files:

```
>sed -n '/^contig\s900\s/, /^contig\s950\s/p; /^contig\s951\s/q'
```

The term ‘contig’ corresponds to the exact name of the reference sequence as used in the file. The first digits (e.g. 900) correspond to the first sequence position to extract. The second number (e.g. 950) indicates the end of the extracted positions. The last number (e.g. 951) explicitly terminates the command execution when this position is reached. By modifying these numbers, regions of interest can be captured in a given assembly and genetic events in this region can be manually verified. With the Linux command ‘>more A.Assem.sort.pileup’ the ‘contig’ term used in a given file can be obtained easily.

3.3.3. Identification of regions of recombination

The ‘FusionReads.pl’ Perl script is aimed at the identification of reads with different parts matching to remote chromosomal regions. When repeatedly spotted within the same chromosomal region, such ‘fusion reads’ are indicators of recombination events. Because the script runs over large ‘.Assem.sam’ files, it may take several minutes to screen all the assemblies. For each assembly, this analysis generates three new files:

- a. ‘.sam_FUSION.POINTS.tsv’ reports counts of fusion reads per contig position.
- b. ‘.sam_FUSION.LINKING.tsv’ assembles reads specifically connecting two remote regions of the reference.

- c. ‘.sam_FUSION.POINTS.fastq’ collects all reads in a ‘.fastq’ format for *de novo* assemblies.

Running the ‘TABmaker.Fusions.pl’ script will gather the files corresponding to different time points. The script generates two files summarizing the counts of fusion reads at different reference positions over the course of evolution (PositionFusions.tsv) and the counts of fusion reads connecting two remote reference regions over the course of evolution.

3.4. Isolation of individual highly tolerant or drug resistant strains

To further characterize mutations acquired during selection, take individual mutant isolates from the frozen stocked populations (step 6 in section 3.1) or generate clean mutants by re-introducing specific mutations identified by sequencing into the isogenic ancestor using standard procedures for *P. aeruginosa* engineering (see **Note 15**). To isolate clonal variants associated with a tolerant or resistant phenotype:

1. Re-streak the frozen stock for single colonies of mutants carrying a specific mutation (step 6 in section 3.1). Pick a number of individual single colonies and verify the presence of individual or combinations of mutations by PCR and DNA sequencing.
2. Inoculate appropriate clones into glass tubes containing 5 mL of LB and incubate on a shaker overnight.
3. Cryopreserve the cultures at -80 °C (see step 6 in section 3.1)
4. Analyze antibiotic-related characteristics of individual clones and/or evolved populations as described in section 3.5.

3.5. Analysis of the antibiotic-related characteristics of individual isolates

To further characterize the contribution of specific mutations to drug tolerance or resistance, analyze the response of each individual mutant strain to antibiotic exposure and compare to a standard curve (see section 3.6).

1. Inoculate the strain to be tested from single colonies into a glass tube containing 5 mL of LB and incubate on a shaker overnight.
2. Determine MIC values of the isolated mutants according to section 3.2.
3. Dilute the overnight culture into 1 mL of LB medium to reach an OD₆₀₀ of 0.24.
4. Prepare a stock solution of the antibiotic in LB medium with a concentration twice the final concentration used in the assay.
5. Mix 100 μ L of bacteria (step 3) with 100 μ L of LB containing drug (step 4) in 96-well plates. Each strain should be tested in triplicate. Incubate the plate at 37 °C, 170 rpm and determine killing curves as a function of time.
6. For each time point, harvest a sample of the culture, make ten-fold serial dilutions in LB and spot 10 μ L of the undiluted and diluted samples on LB agar plates. Incubate plates up to 48 h to determine CFUs per mL of surviving cells. The fraction of survivors is calculated by dividing CFU/mL after treatment by CFU/mL at T₀ (before antibiotic treatment; to determine CFUs at T₀, see step 5 in section 3.1).

3.6. The contribution of low-level resistance to survival and tolerance

Mutations boosting antibiotic tolerance often result in small increases of MIC. At the same time, low level resistance contributes to increased survival even at antibiotic concentrations that far exceed the respective MIC values (20). Thus, when studying tolerant isolates, it is mandatory to monitor resistance values carefully and to clearly distinguish between the contributions of resistance and tolerance to increased survival during antibiotic exposure. As a benchmark, we recommend defining the exact contribution of individual levels of resistance to survival during killing experiments. Spontaneous resistance mutants with well-defined MIC values are first isolated by streaking a low tolerance *P. aeruginosa* lab strain on LB agar plates with defined concentrations of the respective antibiotic (e.g. 2, 4, 8 μ g/ml tobramycin). Isolates with varying levels of resistance can then be tested for survival at high antibiotic

concentrations as described in section 3.5. This experiment produces a standard curve that correlates survival rates with MIC values and that can be used as a baseline to gauge survival benefits originating from *bona fide* tolerance alleles or mechanisms at any given MIC. True tolerance mutations are expected to provide survival benefits way above the MIC standard curve.

1. Inoculate the ancestor strain in a glass tube containing 5 mL of LB and incubate on a shaker overnight.
2. Spot different volumes of the overnight culture onto LB plates containing increasing concentrations of the antibiotic. Plate 100 μ l, 500 μ l and 1-2 mL of bacteria to obtain spontaneous mutants with resistance levels of 2x MIC, 4x MIC or 8-16x MIC, respectively. Incubate plates overnight. If you do not obtain any spontaneous resistance mutants, repeat this step with more bacteria.
3. Pick 10 single colonies from each plate and inoculate clones individually into glass tubes containing 5 mL of LB and incubate overnight.
4. Cryopreserve isolates at -80 °C (see step 6 in section 3.1)
5. Measure MIC of the isolated mutants as described in section 3.2.
6. Store a pellet for genome sequence analysis (see step 7 in section 3.1 and section 3.3).
7. Test the survival of each mutant to antibiotic treatment (step 3-6 in section 3.5)
8. Plot survival scores and MIC in the same graph or survival scores as a function of time for mutants binned based on MIC values.

4. Notes

1. When using antibiotic stock solutions over an extended 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. Plan carefully

and adjust the amount of antibiotic stock solutions and volume of aliquots to the expected needs.

2. Use the same LB batch preparation for the entire evolution experiment to avoid variability due to different medium quality.
3. Make sure to reserve enough sterile Erlenmeyer flasks when starting an evolution experiment. Depending on the turnover of glassware due to cleaning and sterilization at your Institution and depending on the length of the experiment, a 4-5x excess of flasks may be required for the entire experiment.
4. All studies described here used the *P. aeruginosa* PAO1 lab strain as starting point for evolution experiments. All strains should be stored in 15 % DMSO-LB at -80°. Other lab-adapted strains (i.e. PA14) or clinical isolates can be employed similarly. Strains chosen should be sensitive for the antibiotic used.
5. As the objective of the experiment is to provide a comprehensive picture of the bacterial response to specific drug treatments, we suggest to inoculate at least 10 independent lineages in parallel to increase the coverage of the genetic landscape. One should be aware that *P. aeruginosa* lab strains are highly sensitive to tobramycin (see **Note 8**) and that under the assay conditions described here, about 50 % of the initial lineages are aborted due to effective killing.
6. The concentration of aminoglycoside (tobramycin) and fluoroquinolone (ciprofloxacin) stock solutions may need to be adapted depending on the final concentration used in the experiments.
7. For evolution experiments, we recommend using antibiotic concentrations of at least 10x MIC and above the minimal bactericidal concentration. Antibiotic concentrations and durations of treatment should be sufficiently high to achieve a biphasic killing curve but should also allow the regrowth of the surviving population after treatment.

We generally used tobramycin at 32 $\mu\text{g}/\text{mL}$ (32x MIC) and ciprofloxacin at 2.5 $\mu\text{g}/\text{mL}$ (20x MIC) for 3 h. Higher concentrations or longer treatment periods resulted in lineage abortion due to complete eradication during the first treatment. Eradication was not observed with ciprofloxacin, even when used at high concentrations. When tobramycin concentrations below 10 $\mu\text{g}/\text{mL}$ (10x MIC) were used, rapid development of drug resistance was observed (2-3 days). Evolution experiments with drug combinations allowed to reduce tobramycin concentration to 10-16 $\mu\text{g}/\text{mL}$, while ciprofloxacin was generally maintained at 2.5 $\mu\text{g}/\text{mL}$. In general, cell recovery was followed for a period of 48 h after the first and second treatment cycles. If no growth was observed after 48 h, lineages were aborted.

8. For optimal comparison of different evolutionary lineages, we advise to use the same incubator or use incubators with identical properties (e.g. shaking amplitude).
9. By plating droplets, 6 different dilutions can be spotted and analyzed on a single LB plate. Allow plates to dry before use by incubating partially open at 37 °C for a few minutes. Transfer 10 μL of the culture to the agar plate using a multichannel pipette by touching the agar surface with the ends of the 6 tips followed by gentle ejection. Then slightly tilt the plates and allow the droplets to flow towards the opposite side of the plate. Dry the plates for a few seconds.
10. For complete recovery of all viable cells after the antibiotic treatment, plates are incubated for up to 48 h. To avoid that plates are over-grown making cell counting difficult, we recommend counting colonies already after the first overnight incubation and leaving the plates at room temperature for 24 more hours to (re)count slow-growing colonies.
11. In order to properly interpret results from evolution experiments, we advise to always cryopreserve strains from every day of the evolution lineages including the ancestor.

Moreover, we advise to include the ancestor strain of each lineage in the genome sequencing analyses. This will allow distinguishing spontaneous mutations originating *de novo* during the selection experiments from mutations that were already present in the original strain.

12. Survival frequencies after treatment with tobramycin (32 $\mu\text{g}/\text{mL}$) or ciprofloxacin (2.5 $\mu\text{g}/\text{mL}$) were compared with and without an additional LB wash/resuspension step. While significant differences were observed for non-diluted samples, we observed no differences for dilutions equal or greater than 10^{-1} . Therefore, an additional wash/resuspension step was only added when plating undiluted samples.
13. To limit contamination of any kind, all materials should be disinfected when handling different populations. Use micropipette tips with filters and freshly autoclaved solutions of DMSO (15 %, v/v) and LB when handling evolving populations over longer time periods. For the centrifugation step, avoid filling the falcon tubes by pouring the media directly from the flask but instead always use a sterile pipette. Never open two flasks or containers with evolving populations at the same time. Any kind of contamination will result in premature termination of the experiment.
14. We advise to perform the experiment without interruption. If this is not possible, we advise to cryopreserve the overnight cultures and use fresh inoculates from completely thawed cryopreserved backups in case the experiment needs to be started.
15. Isolation of clones with interesting SNPs is important to further characterize the function of the respective genes in antibiotic resilience. We recommend to always reintroduce the SNPs into the original ancestor strain to confirm the role of the specific mutations. Original isolates and re-engineered mutant strains may behave differently despite carrying the same mutation. In such cases, consider epistatic interactions of different mutant alleles in the original isolates.

16. As we perform population genome sequencing, only genetic variations that are observed in a substantial fraction of the sequencing reads (>10 %) of a specific day of the evolution are considered. In many cases, the evolutionary context may be simple enough to infer the presence of sub-lineages by coverage analysis of the population genome sequencing. In other cases, the number of SNPs and their frequency distribution does not allow to infer the possible combinations of the SNPs in a specific clone. In this case, it is advisable to isolate individual clones and always double-check SNPs combinations by PCR.
17. Deep sequencing generally produces several millions of sequenced reads in a single file in Fastq or compressed Fastq.gz formats, both of which are compatible with most downstream applications. An example of a read in Fastq format including the headline with metadata information is shown below with the nucleotide sequence *per se*, a '+' delimiter (can be followed by a repetition of the previous headline) and the ASCII coded base calling quality values for each sequenced nucleotide (one symbol = one score).

```
@D00535:227:CAYRPANXX:8:2209:1825:1964 2:N:0:GCTCATGA+TTATGCCA  
CGGTGATGATGGGCGCCGACTACGTCGAGCCGGACCTGGTGATGACCCGCGACGGCAAGCTGGTCG
```

18. A local alignment is preferred in this particular case as it can be useful to identify reads with different parts matching to remote chromosomal regions. If repeatedly spotted, these “fusion reads” are good indicators of recombination events. Conversely, local alignments might render the identification of single nucleotide polymorphisms more difficult. For example, reads from two similar chromosomal regions that only differ by a nucleotide will be aligned to each region. Later, this might be misinterpreted as two different alleles within the evolving population. However, because

of the time resolution of our approach, only variants arising during evolution are considered. Most of these are factual variants will appear constant and will therefore easily be ruled out.

5. References

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Figure captions

Figure 1. Schematic of the experimental design for the iterative exposure of *P. aeruginosa* to bactericidal antibiotics.

Figure 2. Resistant variants of the *P. aeruginosa* lab strain PAO1 with different MIC were tested for survival when challenged with 32 µg/mL tobramycin. The survival score is plotted as a function of the MIC (**A**) or over time for mutants grouped according to their resistance levels (**B**).