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Bacterial Persistence

Methods and Protocols

Second Edition

Humana Press

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Edited by

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Preface

At the time of this writing, we are in the midst of a global pandemic that presents unprecedented challenges to health systems. Countless lives are lost, and the economic, social, and psychological impact of COVID-19 can hardly be underestimated. This clearly illustrates the devastating power microorganisms can have over human beings. In the case of bacteria, antibiotics have for many years provided an invaluable tool to clear infections and to facilitate surgical procedures and therapies that require immune suppression, including cancer treatments. However, bacteria are developing strategies to circumvent the lethal action of antibiotics at an alarmingly fast pace. In this respect, not only the well-known problem of resistance but also bacterial persistence comes to light. Persister cells comprise a small, transiently multidrug tolerant subpopulation in an otherwise susceptible group of bacteria. These cells contribute to the chronic character of many infections and play a role in the development of genetic resistance, warranting the renewed scientific interest in persistence since the 1980s. However, the transient nature of persister cells and their scarce numbers present a need for dedicated techniques and protocols. This volume combines state-of-the-art methods that are currently used in persistence research and can be instrumental in further deciphering this intriguing phenomenon.

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Part I

Introduction

Chapter 1

Studying Bacterial Persistence: Established Methods and Current Advances

Elen Louwagie, Laure Verstraete, Jan Michiels, and Natalie Verstraeten

Abstract

To date, we are living in a postantibiotic era in which several human pathogens have developed multidrug resistance and very few new antibiotics are being discovered. In addition to the problem of antibiotic resistance, every bacterial population harbors a small fraction of transiently antibiotic-tolerant persister cells that can survive lethal antibiotic attack. Upon cessation of the treatment, these persister cells wake up and give rise to a new, susceptible population. Studies conducted over the past two decades have demonstrated that persister cells are key players in the recalcitrance of chronic infections and that they contribute to antibiotic resistance development. As a consequence, the scientific interest in persistence has increased tremendously and while some questions remain unanswered, many important insights have been brought to light thanks to the development of dedicated techniques. In this chapter, we provide an overview of well-established methods in the field and recent advances that have facilitated the investigation of persister cells and we highlight the challenges to be tackled in future persistence research.

Key words Bacterial persistence, Persistence, Antibiotic tolerance, Antibiotics

1 Bacterial Persistence

In 1942, while studying the mode of action of the just recently discovered penicillin, Hobby and coworkers noted that the killing rate of streptococci decreases over time and that 1% of the bacterial population survives even extended treatment [1]. Two years later, Bigger observed that also a small fraction of staphylococci bacteria withstand penicillin treatment and he called the surviving cells "persisters" [2]. He stated that persisters are in a dormant and nondividing state which is not heritable to the progeny, clearly distinguishing these cells from resistant mutants. While Bigger

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already pointed out that persistence could contribute to the failure of antibiotic treatment, the scientific interest in persister cells remained virtually nonexistent for 40 years following their discovery [2]. Moyed and Bertrand revived attention to persistence research in 1983 with the introduction of the so-called high persistence (hip) mutants [3]. These mutants were originally obtained by chemical mutagenesis and some of them displayed a 10,000-fold increase in persister fraction compared to the parental *Escherichia coli* K-12 strain. The observation of the high-persister phenotype was not restricted to β-lactam antibiotics, indicating for the first time that persistence is not specific to one antibiotic [3]. This incentive, combined with the increasing awareness of the clinical implications of persistence, encouraged other research groups to study persistence, leading to several groundbreaking results over the past years which are summarized in some excellent reviews [4–13]. To date, multiple approaches and experimental setups have been adopted to understand the characteristics of persister cells, resulting in different perspectives on the matter. In 2018, the first international workshop on "Bacterial Persistence and Antimicrobial Therapy" was held in Ascona, Switzerland, and this has led to the publication of a general definition of persistence and guidelines for how to study it [10].

Antibiotic persistence is generally described as the ability of a bacterial population to survive exposure to bactericidal antibiotics. In contrast to population-wide tolerance, this ability is restricted to a small subpopulation of cells within an isogenic population. The killing rate of this isogenic population is thus biphasic with the antibiotic-sensitive subpopulation rapidly being killed, followed by the killing of the antibiotic-tolerant persister subpopulation at a much slower pace (Fig. 1) [10]. Persistence must be distinguished from resistance which is the survival and growth of cells during antibiotic stress due to the acquisition of stable genetic mutations. Persistence is also different from heteroresistance which refers to a subpopulation of cells that survive antibiotic treatment due to their transiently resistant phenotype. Noteworthy, persister cells cannot replicate in the presence of antibiotics while heteroresistant cells can. However, as is the case with heteroresistant cells, the drug insusceptibility of persisters is reversible [14]. Indeed, persister cells can switch back to the antibiotic-sensitive state and resume growth upon antibiotic removal, yielding a new, susceptible population. Persister cells are stochastically generated or formed as a response to environmental changes such as antibiotic or nutrient stress. The exact mechanisms of persister formation are still not clear, although dormancy or inactivity of the antibiotic target have often been linked with persistence. The presence of persister cells has been detected both in vitro and in vivo in several bacterial species [10, 11, 15]. Moreover, a similar phenomenon has been observed

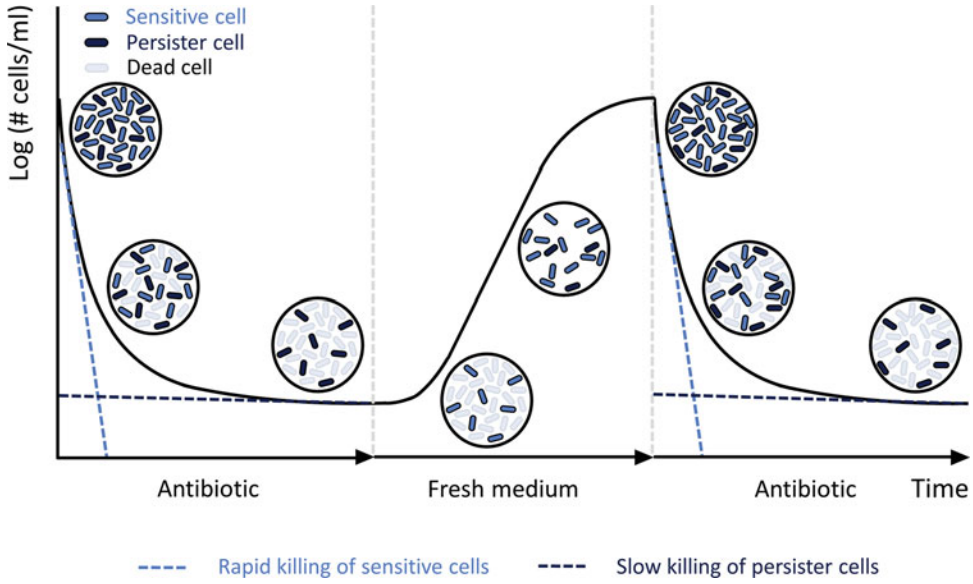

Fig. 1 Survival kinetics of an isogenic population during antibiotic treatment. The bulk population is rapidly killed over time, followed by a slower killing rate due to the presence of antibiotic-tolerant persister cells. Upon removal of the antibiotic and regrowth in fresh medium, persister cells revert to the normal growing state, generating a population that is equally sensitive to antibiotics as the original population

in eukaryotes, which indicates that persistence is a general survival strategy [16, 17].

2 Clinical Relevance

In the past few decades, persister cells have been recognized as key players in the recalcitrance of chronic infections, thereby putting a significant burden on human health [18]. Most of these infections are biofilm-related. For example, *Pseudomonas aeruginosa*, *Candida albicans*, uropathogenic *E. coli*, *Salmonella* species, and *Staphylococcus aureus* are able to attach to the surfaces of organ tissues and/or indwelling devices where they form a biofilm [19–23], that is, a structured microbial community embedded in a self-produced matrix consisting of polysaccharides, lipids, proteins, and DNA [24]. Additionally, uropathogenic *E. coli* is able to invade underlying bladder epithelial cells where it forms quiescent intracellular reservoirs with biofilm-like properties [25]. Within a biofilm environment, pathogens are shielded from the immune system, leaving clearance of the infection to be mostly dependent on antimicrobial treatment. However, effective clearing of the infection is often not achievable with these treatments. For a long time, it was thought that treatment failure was due to impaired penetrance of antimicrobials in the biofilm, but evidence is mounting that biofilms also

harbor an increased number of persister cells that are tolerant to the applied antimicrobials [16, 26–30]. A first correlation was shown ten years ago through the identification of hip mutants amongst clinical isolates originating from cystic fibrosis patients with chronic *P. aeruginosa* infections [31] and patients with chronic *C. albicans* infections [32]. Recently, similar evidence was provided for recurrent urinary tract infections caused by uropathogenic *E. coli* [33]. Persister cells have also been shown to be important in non–biofilm-related chronic infections. Well-known examples are *Mycobacterium tuberculosis* infections, in which *M. tuberculosis* invades and reprograms alveolar macrophages and resides in a tolerant, dormant state inside granulomas, while being protected from immune responses [34], a strategy that is also used by *Salmonella* species [35]. Recently, a screen of *M. tuberculosis* clinical isolates revealed the presence of hip mutants [36]. Besides being responsible for the recalcitrant nature of important chronic infections, persistence has been shown to pave the way for resistance development, which is recognized as one of the biggest threats to human health [37]. Both the prolonged presence of the infecting pathogen under intermittent antibiotic treatment and the increased mutation rates associated with the persister phenotype enhance the development of resistance-conferring mutations [38–40]. Finally, parallels have been drawn between microbial persistence and the presence of tolerant subpopulations in cancer tumors [41–45]. Altogether, persistence plays a pivotal role, not only in chronic infections, but also in resistance development and the outcome of cancer treatments, which demonstrates the importance of persistence research.

3 Challenges in Persistence Research

Although recent years have seen a tremendous increase in persistence research, the interest in the phenomenon is of a relatively new nature. Not only has the clinical relevance of persistence remained highly underestimated for many years, the study of persister cells has also been impeded by the many difficulties that are inherently linked to the phenotype. The continuous switching between the normal and persister state gives rise to a variable yet usually very small persister fraction, typically within a range of 0.001% to 1% of the entire population [9]. Moreover, this subpopulation is characterized by heterogeneity in cell physiologies and tolerance to antibiotics [46]. These issues have been tackled by major advances in microscopic and omics technologies, and the enrichment and isolation of persisters. New microscopic techniques allow researchers to study persistence at the single-cell level using fluorescent (time-lapse) microscopy, microfluidics, and flow cytometry, which yields valuable information on cell growth and cell death, metabolic state and gene expression of individual cells [14]. In these single-cell

studies, as well as in proteome and transcriptome analyses, the enrichment and isolation of persisters is crucial given the low number of persister cells. One commonly used approach is to lyse all antibiotic-sensitive cells by antibiotic treatment and harvest the nonlysed, antibiotic-tolerant persister cells by centrifugation [47, 48] or the use of magnetic beads [49]. These methods rely on the killing kinetics of antibiotics and thus on the physiological state of the bacteria which is circumvented by lysing normal cells using a mixture of alkaline and enzymatic solutions [50]. Another approach is based on the assumption that persister cells are in a dormant, nongrowing state and/or that growth-related genes are not, or to a lesser extent, expressed in persister cells. By the use of fluorescent reporters such as the green fluorescent protein (GFP), persister cells can be sorted from the bulk population by fluorescence activated cell sorting (FACS) [51] or quantified by flow cytometry [52–55]. New isolation and enrichment protocols are based on the filamentation of β-lactam-treated bacteria followed by filtration to isolate persister cells [56] or cyclic exposure to antibiotics [57]. Prior to isolation, the formation of persister-like cells can be induced by antibiotic or chemical pretreatment [58, 59] or toxin overexpression [60]. Since the persister population is heterogeneous and variable, and most enrichment and isolation methods rely on persister physiology, persistence should be studied by a combination of population and single-cell approaches which both will benefit from future technological advances. Currently used methods and recent advances to study persistence are briefly introduced below.

4 Current Methods

4.1 Genetic Screenings

One of the first methods to be developed for the identification of new persister mechanisms was the large-scale screening of transposon, knockout, and overexpression libraries. In general, strains from a mutant library are exposed to antibiotics for several hours and the number of surviving cells is determined. Strains with an increased or decreased survival presumably contain one or multiple mutated or overexpressed genes involved in persistence and are further tested for sensitivity to other antibiotics and antibiotic resistance. Two research groups have independently screened *E. coli* mini-Tn*10* insertion libraries, both times yielding a few knockout mutants with reduced persister levels upon antibiotic exposure [61, 62]. Also in other organisms such as *M. tuberculosis*, *P. aeruginosa*, and *S. aureus*, transposon mutagenesis has been performed to gain new insights in general persistence pathways [63–66]. The *E. coli* K-12 knockout library (Keio collection) [67] has also been subjected to persistence screening by two independent groups using different antibiotics and growth conditions, leading to

the identification of several novel persister genes [68, 69]. The abovementioned screenings allowed to identify only nonessential genes and their potential role in persistence. To discover genes that are indispensable for growth as well as regulatory RNAs with a role in persistence, screening of an overexpression library has proven instrumental [68, 70]. Overexpression of two proteins involved in glycerol metabolism resulted in an increase of persister survival in *E. coli* [71]. Other gain-of-function screenings also showed a link between energy production and increased persistence [72, 73]. Finally, due to recent advances in next-generation sequencing, Tn-Seq [74] and Barseq [17] have emerged as popular high-throughput screening methods in the search for new persister genes.

While genetic screenings have led to the discovery of many new persister genes, the role of these genes in persister formation and/or maintenance is in many cases still not clear. Importantly, the genome-wide screenings revealed mostly global regulators and genes involved in cell metabolism and did not produce any mutants unable to form persisters. This strongly suggests that there is not just one single mechanism underlying persistence and that several molecular pathways are involved.

4.2 Experimental Evolution

Several studies have shown that persistence can increase the fitness of the entire bacterial population in stressful conditions [75–77] and could thus be a phenotypic trait that can be selected for. Indeed, evolution experiments have shown that the number of persister cells can be tuned by the frequency of antibiotic exposure. In these experiments, bacterial cultures are intermittently exposed to high-dose antibiotics and regrown upon suspension in fresh medium, reflecting the clinical settings in which patients are regularly treated with antibiotics. It is therefore assumed that the studied persister cells and associated mutations are similar to those encountered in natural environments. Experimental evolution was already performed by Moyed and Bertrand in 1983 when they periodically treated *E. coli* populations with ampicillin, resulting in the evolution of hip mutants including the *hipA7* strain that has since been widely used in persister research (*see* e.g. Subheadings 4.3.1 and 4.4.1 below) [3]. Later, two important evolution experiments were performed showing a rapid evolution toward increased persister levels [78] and tolerance [79] in *E. coli* without antibiotic resistance development. Different conditions (e.g., growth phase of the population prior to treatment, antibiotics, treatment duration) were used in these studies, but both experiments showed that in some of the evolved clones only a single point mutation was responsible for the increase in survival [78, 79]. Noteworthy, elevated persister levels in the notorious ESKAPE pathogens [80] and evolution to tolerance in *S. aureus* [81] upon periodic application of antibiotics were also observed. Some recent evolution experiments were also combined with proteomics or transcriptomics

analyses, allowing to study the proteome and transcriptome of the evolved mutants [57, 82]. Finally, experimental evolution has been used to study the link between persistence and resistance [39, 83].

4.3 Transcriptomics and Proteomics

In addition to genetic screenings and experimental evolution, transcriptomics and proteomics have found their way to persistence research. This is not surprising, as persister cells are phenotypic variants within an isogenic population and thus differ from the majority of the population in transcript and protein contents. However, as the persister population comprises a small fraction of the total population, their transcriptome and proteome profiles can only be obtained after increasing the persister fraction and/or after proper enrichment or isolation of persister cells.

4.3.1 Transcriptomics

In 2004, the Lewis group was the first to study the expression profile of persister cells [47]. To cope with the low abundance of persisters, experiments were conducted with an *E. coli* strain carrying the *hipA7* allele. To enrich the persister cells for transcriptome analysis, an exponential-phase culture was treated with the β-lactam antibiotic ampicillin and nonlysed persister cells were isolated through centrifugation. RNA was extracted from the isolated persister cells, enriched for mRNA, converted to cDNA, hybridized to a microarray and analyzed. The expression profile of persister cells showed a downregulation of metabolism and flagellar synthesis pathways and an upregulation of toxin–antitoxin (TA) modules and other proteins that slow down active bacterial processes such as translation and replication, all pointing toward a dormant state [47]. However, it should be noted that this isolation method has its downsides, as the antibiotic treatment prior to isolation could affect the expression profile. A few years later, the same research group exploited its findings to develop an alternative method for persister isolation from a wild-type *E. coli* population, without involvement of antibiotic treatment, that is based on the assumption of persister cells being dormant and thus having their translation machinery shut down [51]. Briefly, a degradable GFP was placed under the control of a ribosomal promoter and dim, growth-arrested cells were isolated using FACS. This isolation method proved its effectiveness, as the dim population was shown to be tolerant to antibiotics. Next, the expression profile of the dim persister population was determined as described before, which confirmed their previous findings [47, 51]. In 2011, the Lewis group also succeeded in determining the expression profile of *M. tuberculosis* exponential-phase persisters using similar techniques [84]. The method used to isolate persisters was again based on lysis of nonpersister cells but now using the cell wall-acting antibiotic D-cycloserine, followed by centrifugation. This study showed the expression profile of *M. tuberculosis* persisters to be very similar to the one of *E. coli* persisters [84]. Microarray hybridization was also used by the

Michiels group to investigate the role of DnpA, a de-*N*-acetylase, in *P. aeruginosa* persister formation by comparing expression profiles of the wild-type strain, a *dnpA* mutant and a *dnpA* overexpression strain, of which the latter causes an increased persister fraction. From this, the authors learnt that *dnpA* overexpression coincides with decreased amino acid biosynthesis and metabolism, which is in accordance with the studies mentioned above [85]. Later, transcriptome profiling of persisters was also done in other microorganisms, but then using the state-of-the-art RNA-sequencing technique instead of microarray hybridization [86–88]. Here, a library of cDNA fragments with ligated adapters is generated and subsequently sequenced using one of the currently existing high-throughput sequencing platforms, such as Illumina [89]. In a study with *Staphylococcus epidermidis*, RNA-seq analysis of biofilms containing different portions of dormant bacteria revealed that mostly genes related to oxidation-reduction processes and acetyl-CoA metabolic reactions are differently expressed in persister cells [86]. The same technology was also used to compare the expression profiles of *Listeria monocytogenes* nisin-treated and untreated cultures, showing persister cells to have a decreased energy production and flagellar synthesis [87]. Finally, RNA-seq analysis of macrophages infected with *Salmonella* Typhimurium revealed that residing persister cells have indeed ceased growth, but instead of being completely dormant, they still show transcriptional and translational activity enabling them to reprogram their macrophage host [88].

4.3.2 Proteomics

Proteome profiling of persister cells holds great promise to gain insight in the persister phenotype because it allows for protein identification and quantification as well as the identification of interaction partners and post-translational modifications, which could reveal hidden levels of regulation. However, as standard proteomic analyses require high cell numbers and the number of persister cells in a culture is usually low, these types of studies are especially challenging. This explains why the number of reports applying proteomics to persister populations is rather limited. Moreover, the few studies that have been conducted have focused almost exclusively on *M. tuberculosis* and *C. albicans* persister cells due to their abundant presence inside granulomas and biofilms, respectively, and their ease of isolation [90]. *M. tuberculosis* resides in a nonreplicating persistent state inside granulomas, an environment that is characterized by hypoxia and nutrient starvation [91]. By imposing 6 weeks of nutrient starvation, these conditions were mimicked in vitro and then the proteome of both log-phase and nutrient-starved cells was analyzed through two-dimensional gel electrophoresis combined with liquid chromatography tandem mass spectrometry (LC-MS/MS) and/or matrix-assisted laser

desorption/ionization MS (MALDI-MS). These analyses revealed an increased abundance of TA modules, lipoproteins, and stringent response effectors and a decreased abundance of proteins involved in energy metabolism and amino acid, protein, and lipid biosynthesis [92, 93], thereby complementing the results obtained through transcriptomics studies [84]. In 2015, two research groups induced the nonreplicating persistent state by applying hypoxia and subsequently analyzed the proteome profile of these *M. tuberculosis* cells, as well as of reoxygenated, reactivated cells and exponential-phase cells grown in normoxic conditions, through LC-MS/MS or SWATH-MS [94, 95], thereby confirming previous findings [92, 93]. In addition to *M. tuberculosis* granulomas, *C. albicans* biofilms have also been shown to harbor an increased number of persister cells [16, 96–98], although it should be noted that two studies are now contradicting this statement [99, 100]. Similar to studies conducted in *M. tuberculosis*, LC-MS/MS was used to investigate the proteome of persister-enriched *C. albicans* biofilm populations, revealing an overall downregulation of metabolic processes, an upregulation of some genes related to the glyoxylate pathway, growth, virulence and the stress response [96], and a crucial role for alkyl hydroperoxide reductase 1 (AHP1) in persistence against amphotericin B [97]. Although challenging, methods have recently been developed to determine the proteomic profile of persisters in other microorganisms, such as *E. coli*. As their abundance is typically low, the number of persister cells should be increased or persister cells should be isolated prior to proteome analysis. As such, the proteome of nutrient-shift-induced *E. coli* persister cells could be investigated [101]. In another study, the persister fraction of *E. coli* populations was increased through evolution under cyclic antibiotic treatment and then proteomic analysis was performed on the evolved stationary-phase populations [57].

4.4 Single-cell Techniques

Given the low abundance of persister cells and the transient and heterogeneous nature of the persister phenotype, persistence research greatly benefits from existing single-cell techniques such as single-cell time-lapse microscopy and flow cytometry.

4.4.1 Single-cell Time-Lapse Microscopy

Single-cell time-lapse microscopy allows to monitor the behavior of single cells over time through microscopic image analysis. In a pioneering study, Balaban [102] were the first to image single cells of an *E. coli* bacterial culture in a microfluidics device through the phases of treatment, in which the sensitive cells of the population were lysed through the action of ampicillin, followed by persister awakening and outgrowth in fresh medium. To avoid cells overgrowing each other and thus loosing track of cells, the device was patterned with narrow grooves in which the cells were loaded [102]. To cope with the issue of low abundance of persister cells,

the experiments were conducted with a *hipA7* mutant. This system allowed the authors to build a mathematical model describing the switching rates between sensitive and persister cells [102]. Afterward, microfluidic devices have also been used to follow fluorescent protein induction in both sensitive and persister cells upon addition of fresh medium, before and after treatment, to further characterize the dormant persister state [103], to study the role of indole in persister formation [104], and to study the awakening mechanism of HokB-induced persister cells [105]. Despite the different strategies that are being used to increase the number of persister cells and the persister isolation methods that have been developed, a major downside of single-cell time-lapse microscopy of persister cells remains its relatively low throughput. Moreover, the existing microfluidic configurations do not allow to select and sort out specific single cells for further investigation.

4.4.2 Flow Cytometry

These throughput and sorting issues are circumvented by flow cytometry, a method that allows to assay thousands or even millions of single cells based on fluorescent measurements, followed by FACS to sort out the cells of interest. Although flow cytometry does not allow to track single cells over time, it has been proven very useful to study the persister phenotype. First of all, flow cytometry can be used to show the presence of a persister population within a culture. For example, *E. coli* stationary-phase populations were investigated with flow cytometry using a GFP dilution method and the presence of a bright, nongrowing persister population was confirmed [52]. Similarly, dual DsRed-GFP dilution and TIMER^{bac} were used to study *Salmonella* subpopulations [53, 54]. Importantly, not only can persister populations be identified, but they can also be discerned from the viable but nonculturable (VBNC) cells within a population [55]. Second, persister awakening kinetics can be deduced from flow cytometry data using these fluorescent protein dilution methods [55, 106, 107]. Third, flow cytometry combined with FACS has become the state-of-the-art technique to study persister physiology. For example, by using the metabolic dye redox sensor green, it was shown that dormancy is not sufficient nor required for a cell to become a persister cell [108]. Alternatively, persister physiology can be studied by using fluorescent reporter fusions and screening the sorted populations for persistence [51, 109, 110]. Lastly, as already mentioned earlier, FACS can be used for isolation of persister cells [51].

4.4.3 Advances in Single-cell Technologies

To date, there has only been one study combining high-throughput and the ability to track and recover cells of interest. In this study, a carbenicillin-treated *P. aeruginosa* culture resuspended and diluted in fresh medium was applied to a hydrophilic-in-hydrophobic patterned array, resulting in 10^6 dome-shaped

femtoliter droplets, each containing maximally one persister cell. After overnight incubation of the array, the cells that had divided were picked up using an automated micropipette and were checked for antibiotic susceptibility to exclude antibiotic resistance development [111, 112]. It is the rise of such innovative technologies that will provide great potential for future persistence research.

4.5 Mathematical Modelling

The outcome of wet lab experiments greatly depends on the used experimental procedures, often resulting in discrepancies in reported results when different methods are used. This is especially true for persistence as it is a complex phenotype to detect, quantify and study in detail [10, 113]. A major advantage of modelling is that wet lab experiments are strictly not necessary but rather used to support experimental outcomes or test new hypotheses. Moreover, predictive models can take into account clinically relevant antibiotic treatment profiles [40, 114] and the effect of the immune system and other host factors [115]. Two major types of models have been implemented in persistence research: population models, which describe the dynamics of populations and subpopulations, and mechanistic models, which focus on molecular mechanisms at the single-cell level. One of the first population models by Balaban [102] described the stochastic switching between normal and persister cells assuming constant rates of persister cell formation and awakening [102]. Later studies have used and extended this basic model to predict persister levels in different fluctuating conditions [75, 116, 117]. Interestingly, these models show that persister levels change according to the frequency of the antibiotic treatment [75, 78, 116–118], which is in line with empirical observations [78, 80]. An alternative population model proposes the asymmetric ageing of bacteria as a mechanism to explain the presence of persister cells [119]. More recent research focuses on the prediction of antibiotic resistance development from a persister population [39, 40, 114, 120]. Mechanistic models, on the other hand, describe the mechanisms responsible for the coexistence of normal, antibiotic-sensitive cells and antibiotic-tolerant cells within an isogenic population. These models explain phenotypic bistability by the stochastic expression of genes linked with persister formation and resuscitation, which is amplified by feedback loops. For example, the differential expression of toxin–antitoxin modules has been extensively studied in the past (e.g., [121–124]). However, the complete regulatory network is not unraveled yet and the identification of novel persister genes is essential to explain the observed phenotypic variability by mechanistic models.

Even though predictive models provide us with crucial insights in persistence, most models focus on stochastic switching of persisters while the switching in response to environmental stimuli is often not considered. This leaves a gap in the current mathematical

predictions. Moreover, additional in vitro and in vivo observations are required as they could improve existing models [125].

4.6 In vivo Models

A key question in persistence research is whether in vitro and in silico findings can be extrapolated to in vivo settings. In vivo models are not only interesting to study persister mechanisms but could also be a direct proof of the role of persister cells in relapsing infections. Several research groups have developed intracellular, biofilm, and chronic infection models to study persistence and most of the models are discussed in the review of Van den Bergh et al (2017) [9]. In some of these models, persister determinants identified in vitro could be validated. For example, a *P. aeruginosa* Δ*relA spoT* mutant displays lower persister levels compared to the wild type upon treatment with ofloxacin in laboratory conditions. Similarly, infection by this mutant in murine intraperitoneal and subcutaneous biofilm infection models results in increased ofloxacin activity as exemplified by the increased mice survival and lower CFU counts, respectively [126]. However, some of the animal studies show discrepancies between in vitro and in vivo results. A *Salmonella* Δ*lon* mutant, for example, does not alter the persistent phenotype in vitro, while infection by the same mutant in an intracellular macrophage model results in reduced persister formation compared to the wild-type strain [35]. Similarly, mutations that increase and decrease persistence in vivo were identified by infecting mice with a *M. tuberculosis* transposon library, followed by isoniazid treatment. These mutations, however, did not alter in vitro persister levels [127]. These examples show that in vivo models are crucial to study complex and condition-dependent phenotypes such as persistence. In addition to elucidating the characteristics and clinical relevance of persistence, in vivo models have been used to test the efficacy of new antipersister therapies [127–135], although animal models to specifically evaluate potential compounds do not exist yet.

5 Future Perspectives

The methods described above have all contributed to the current knowledge of persistence. However, all of these techniques have their advantages and disadvantages and many questions remain unanswered. Given the challenges inherent to persistence research, that is, the heterogeneous and stochastic nature of the persister phenotype, and the shortcomings of the current persister isolation methods, there is a clear need to explore and implement high-throughput, single-cell techniques and to optimize representative in vivo models. Future advances in persistence methodologies will lead to new insights, thereby enhancing the development of antipersister therapies. The latter will not only allow effective treatment

of otherwise chronic infections, but will also slow down resistance development which will be crucial in the postantibiotic era we live in.

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References

1. Hobby GL, Meyer K, Chaffee E (1942) Observations on the mechanism of action of penicillin. Exp Biol Med 50:281–285
2. Bigger JW (1944) Treatment of staphylococcal infections with penicillin. Lancet 244:497–500
3. Moyed HS, Bertrand KP (1983) *hipA*, a newly recognized gene of *Escherichia coli* K-12 that affects frequency of persistence after inhibition of murein synthesis. J Bacteriol 155:768–775
4. Lewis K (2010) Persister cells. Annu Rev Microbiol 64:357–372
5. Amato SM, Fazen CH, Henry TC et al (2014) The role of metabolism in bacterial persistence. Front Microbiol 5:70
6. Harms A, Maisonneuve E, Gerdes K (2016) Mechanisms of bacterial persistence during stress and antibiotic exposure. Science 354: aaf4268
7. Brauner A, Fridman O, Gefen O, Balaban NQ (2016) Distinguishing between resistance, tolerance and persistence to antibiotic treatment. Nat Rev Microbiol 14:320–330
8. Radzikowski JL, Schramke H, Heinemann M (2017) Bacterial persistence from a system-level perspective. Curr Opin Biotechnol 46:98–105
9. Van den Bergh B, Fauvart M, Michiels J (2017) Formation, physiology, ecology, evolution and clinical importance of bacterial persisters. FEMS Microbiol Rev 41:219–251
10. Balaban NQ, Helaine S, Lewis K et al (2019) Definitions and guidelines for research on antibiotic persistence. Nat Rev Microbiol 17:441–448
11. Wilmaerts D, Windels EM, Verstraeten N, Michiels J (2019) General mechanisms leading to persister formation and awakening. Trends Genet 35:401–411
12. Gollan B, Grabe G, Michaux C, Helaine S (2019) Bacterial persisters and infection: past, present, and progressing. Annu Rev Microbiol 73:359–385
13. Bakkeren E, Diard M, Hardt W-D (2020) Evolutionary causes and consequences of bacterial antibiotic persistence. Nat Rev Microbiol 18:479–490
14. Dewachter L, Fauvart M, Michiels J (2019) Bacterial heterogeneity and antibiotic survival: understanding and combatting persistence and heteroresistance. Mol Cell 76:255–267
15. Lewis K (2007) Persister cells, dormancy and infectious disease. Nat Rev Microbiol 5:48–56
16. LaFleur MD, Kumamoto CA, Lewis K (2006) *Candida albicans* biofilms produce antifungal-tolerant persister cells. Antimicrob Agents Chemother 50:3839–3846
17. Bojsen R, Regenberg B, Gresham D, Folkesson A (2016) A common mechanism involving the TORC1 pathway can lead to amphotericin B-persistence in biofilm and planktonic *Saccharomyces cerevisiae* populations. Sci Rep 6:21874
18. Fauvart M, De Groote VN, Michiels J (2011) Role of persister cells in chronic infections: clinical relevance and perspectives on anti-persister therapies. J Med Microbiol 60:699–709
19. Douglas LJ (2003) *Candida* biofilms and their role in infection. Trends Microbiol 11:30–36
20. Steenackers H, Hermans K, Vanderleyden J, De Keersmaecker SCJ (2012) *Salmonella* biofilms: an overview on occurrence, structure, regulation and eradication. Food Res Int 45:502–531

21. Mulcahy LR, Isabella VM, Lewis K (2014) *Pseudomonas aeruginosa* biofilms in disease. Microb Ecol 68:1–12
22. Speziale P, Pietrocola G, Foster TJ, Geoghegan JA (2014) Protein-based biofilm matrices in Staphylococci. Front Cell Infect Microbiol 4:171
23. Narayanan A, Nair MS, Muyyarikkandy MS, Amalaradjou MA (2018) Inhibition and inactivation of uropathogenic *Escherichia coli* biofilms on urinary catheters by sodium selenite. Int J Mol Sci 19:1703
24. Flemming H-C, Wingender J (2010) The biofilm matrix. Nat Rev Microbiol 8:623–633
25. Anderson GG, Martin SM, Hultgren SJ (2004) Host subversion by formation of intracellular bacterial communities in the urinary tract. Microbes Infect 6:1094–1101
26. Lewis K (2001) Riddle of biofilm resistance. Antimicrob Agents Chemother 45:999–1007
27. Lewis K (2005) Persister cells and the riddle of biofilm survival. Biochemist 70:267–274
28. Roberts ME, Stewart PS (2005) Modelling protection from antimicrobial agents in biofilms through the formation of persister cells. Microbiology 151:75–80
29. Spoering AL, Lewis K (2001) Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. J Bacteriol 183:6746–6751
30. Waters EM, Rowe SE, O'Gara JP, Conlon BP (2016) Convergence of *Staphylococcus aureus* persister and biofilm research: can biofilms be defined as communities of adherent persister cells? PLoS Pathog 12:e1006012
31. Mulcahy LR, Burns JL, Lory S, Lewis K (2010) Emergence of *Pseudomonas aeruginosa* strains producing high levels of persister cells in patients with cystic fibrosis. J Bacteriol 192:6191–6199
32. LaFleur MD, Qi Q, Lewis K (2010) Patients with long-term oral carriage harbor high-persister mutants of *Candida albicans*. Antimicrob Agents Chemother 54:39–44
33. Schumacher MA, Balani P, Min J et al (2015) HipBA-promoter structures reveal the basis of heritable multidrug tolerance. Nature 524:59–64
34. Gengenbacher M, Kaufmann SHE (2012) *Mycobacterium tuberculosis*: success through dormancy. FEMS Microbiol Rev 36:514–532
35. Helaine S, Cheverton AM, Watson KG et al (2014) Internalization of *Salmonella* by macrophages induces formation of nonreplicating persisters. Science 343:204–208
36. Torrey HL, Keren I, Via LE et al (2016) High persister mutants in *Mycobacterium tuberculosis*. PLoS One 11:e0155127
37. O'Neill J (2016) Tackling drug-resistant infections globally: final report and recommendations: the review on antimicrobial resistance. Available via https://www.biomerieuxconnection.com/wp-content/uploads/2018/04/Tackling-Drug-Resistant-Infections-Globally_-Final-Report-and-Recommendations.pdf. Accessed 26 August 2020
38. Cohen NR, Lobritz MA, Collins JJ (2013) Microbial persistence and the road to drug resistance. Cell Host Microbe 13:632–642
39. Levin-Reisman I, Ronin I, Gefen O et al (2017) Antibiotic tolerance facilitates the evolution of resistance. Science 355:826–830
40. Windels EM, Michiels JE, Fauvart M et al (2019) Bacterial persistence promotes the evolution of antibiotic resistance by increasing survival and mutation rates. ISME 13:1239–1251
41. Sharma SV, Lee DY, Li B et al (2010) A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. Cell 141:69–80
42. Chen J, Li Y, Yu TS et al (2012) A restricted cell population propagates glioblastoma growth after chemotherapy. Nature 488:522–526
43. Raha D, Wilson TR, Peng J et al (2014) The cancer stem cell marker aldehyde dehydrogenase is required to maintain a drug-tolerant tumor cell subpopulation. Cancer Res 74:3579–3590
44. Pribluda A, De La Cruz CC, Jackson EL (2015) Intratumoral heterogeneity: from diversity comes resistance. Clin Cancer Res 21:2916–2923
45. Terai H, Kitajima S, Potter DS et al (2018) ER stress signaling promotes the survival of cancer "persister cells" tolerant to EGFR tyrosine kinase inhibitors. Cancer Res 78:1044–1057
46. Allison KR, Brynildsen MP, Collins JJ (2011) Heterogeneous bacterial persisters and engineering approaches to eliminate them. Curr Opin Microbiol 14:593–598
47. Keren I, Shah D, Spoering A et al (2004) Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. J Bacteriol 186:8172–8180
48. Pu Y, Zhao Z, Li Y et al (2016) Enhanced efflux activity facilitates drug tolerance in dormant bacterial cells. Mol Cell 62:284–294

49. Sulaiman JE, Hao C, Lam H (2018) Specific enrichment and proteomics analysis of *Escherichia coli* persisters from rifampin pretreatment. J Proteome Res 17:3984–3996
50. Cañas-Duarte SJ, Restrepo S, Pedraza JM (2014) Novel protocol for persister cells isolation. PLoS One 9:e88660
51. Shah D, Zhang Z, Khodursky A et al (2006) Persisters: a distinct physiological state of *E. coli*. BMC Microbiol 6:53
52. Roostalu J, Jõers A, Luidalepp H et al (2008) Cell division in *Escherichia coli* cultures monitored at single cell resolution. BMC Microbiol 8:68
53. Helaine S, Thompson JA, Watson KG et al (2010) Dynamics of intracellular bacterial replication at the single cell level. Proc Natl Acad Sci U S A 107:3746–3751
54. Claudi B, Spröte P, Chirkova A et al (2014) Phenotypic variation of *Salmonella* in host tissues delays eradication by antimicrobial chemotherapy. Cell 158:722–733
55. Golam S, Kavousi P, Orman MA (2020) Flow-cytometry analysis reveals persister resuscitation characteristics. BMC Microbiol 20:202
56. Windels EM, Meriem Z Ben, Zahir T et al (2019) Enrichment of persisters enabled by a ß-lactam-induced filamentation method reveals their stochastic single-cell awakening. Commun Biol 2:426
57. Sulaiman JE, Lam H (2020) Proteomic investigation of tolerant *Escherichia coli* populations from cyclic antibiotic treatment. J Proteome Res 19:900–913
58. Dörr T, Lewis K, Vulić M (2009) SOS response induces persistence to fluoroquinolones in *Escherichia coli*. PLoS Genet 5: e1000760
59. Johnson PJT, Levin BR (2013) Pharmacodynamics, population dynamics, and the evolution of persistence in *Staphylococcus aureus*. PLoS Genet 9:e1003123
60. Mok WWK, Park JO, Rabinowitz JD, Brynildsen MP (2015) RNA futile cycling in model persisters derived from MazF accumulation. mBio 6:e01588-15
61. Li Y, Zhang Y (2007) PhoU is a persistence switch involved in persister formation and tolerance to multiple antibiotics and stresses in *Escherichia coli*. Antimicrob Agents Chemother 51:2092–2099
62. Hu Y, Coates ARM (2005) Transposon mutagenesis identifies genes which control antimicrobial drug tolerance in stationary-phase *Escherichia coli*. FEMS Microbiol Lett 243:117–124
63. Dhar N (2010) Mckinney JD. *Mycobacterium tuberculosis* persistence mutants identified by screening in isoniazid-treated mice 107:12275–12280
64. De Groote VN, Verstraeten N, Fauvart M et al (2009) Novel persistence genes in *Pseudomonas aeruginosa* identified by high-throughput screening. FEMS Microbiol Lett 297:73–79
65. Manuel J, Zhanel GG, De Kievit T (2010) Cadaverine suppresses persistence to carboxypenicillins in *Pseudomonas aeruginosa* PAO1. Antimicrob Agents Chemother 54:5173–5179
66. Wang W, Chen J, Chen G et al (2015) Transposon mutagenesis identifies novel genes associated with *Staphylococcus aureus* persister formation. Front Microbiol 6:1437
67. Baba T, Ara T, Hasegawa M et al (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol 2:2006.0008
68. Hansen S, Lewis K, Vulić M (2008) Role of gobal regulators and nucleotide metabolism in antibiotic tolerance in *Escherichia coli*. Antimicrob Agents Chemother 52:2718–2726
69. Ma C, Sim S, Shi W et al (2010) Energy production genes *sucB* and *ubiF* are involved in persister survival and tolerance to multiple antibiotics and stresses in *Escherichia coli*. FEMS Microbiol Lett 303:33–40
70. Lewis K (2008) Multidrug tolerance of biofilms and persister cells. In: Romeo T (ed) bacterial bioflms. Current topics in microbiology and immunology, vol 322. Springer, Heidelberg
71. Spoering AL, Vulić M, Lewis K (2006) GlpD and PlsB participate in persister cell formation in *Escherichia coli*. J Bacteriol 188:5136–5144
72. Leung V, Lévesque CM (2012) A stress-inducible quorum-sensing peptide mediates the formation of persister cells with noninherited multidrug tolerance. J Bacteriol 194:2265–2274
73. Girgis HS, Harris K, Tavazoie S (2012) Large mutational target size for rapid emergence of bacterial persistence. Proc Natl Acad Sci U S A 109:12740–12745
74. Shan Y, Lazinski D, Rowe S et al (2015) Genetic basis of persister tolerance to aminoglycosides in *Escherichia coli*. mBio 6:e00078-15
75. Kussell E, Kishony R, Balaban NQ, Leibler S (2005) Bacterial persistence: a model of survival in changing environments. Genetics 169:1807–1814
76. Kussell E, Leibler S (2005) Phenotypic diversity, population growth, and information in

fluctuating environments. Science 309:2075–2078

77. Stewart B, Rozen DE (2011) Genetic variation for antibiotic persistence in *Escherichia coli*. Evolution 66:933–939
78. Van den Bergh B, Michiels JE, Wenseleers T et al (2016) Frequency of antibiotic application drives rapid evolutionary adaptation of *Escherichia coli* persistence. Nat Microbiol 1:16020
79. Fridman O, Goldberg A, Ronin I et al (2014) Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. Nature 513:418–421
80. Michiels JE, Van den Bergh B, Verstraeten N, Fauvart M (2016) *In vitro* emergence of high persistence upon periodic aminoglycoside challenge in the ESKAPE pathogens. Antimicrob Agents Chemother 60:4630–4637
81. Mechler L, Herbig A, Paprotka K et al (2015) A novel point mutation promotes growth phase-dependent daptomycin tolerance in *Staphylococcus aureus*. Antimicrob Agents Chemother 59:5366–5376
82. Khare A, Tavazoie S (2020) Extreme antibiotic persistence via heterogeneity-generating mutations targeting translation. mSystems 5: e00847-19
83. Windels EM, Michiels JE, Bergh B Van Den, Fauvart M (2019) Antibiotics: combatting tolerance to stop resistance. mBio 10: e02095–19
84. Keren I, Minami S, Rubin E, Lewis K (2011) Characterization and transcriptome analysis of *Mycobacterium tuberculosis* persisters. mBio 2: e00100-11
85. Liebens V, Defraine V, Ven der Leyden A et al (2014) A putative de-*N*-acetylase of the PIG-L superfamily affects fluoroquinolone tolerance in *Pseudomonas aeruginosa*. Pathog Dis 71:39–54
86. Carvalhais V, França A, Cerca F et al (2014) Dormancy within *Staphylococcus epidermidis* biofilms: a transcriptomic analysis by RNA-seq. Appl Microbiol Biotechnol 98:2585–2596
87. Wu S, Yu P-L, Wheeler D, Flint S (2018) Transcriptomic study on persistence and survival of *Listeria monocytogenes* following lethal treatment with nisin. J Glob Antimicrob Resist 15:25–31
88. Stapels DAC, Hill PWS, Westermann AJ et al (2018) *Salmonella* persisters undermine host immune defenses during antibiotic treatment. Science 362:1156–1160
89. Wang Z, Gerstein M, Snyder M (2009) RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet 10:57–63
90. Sulaiman JE, Lam H (2019) Application of proteomics in studying bacterial persistence. Expert Rev Proteomics 16:227–239
91. Wayne LG (1994) Dormancy of *Mycobacterium tuberculosis* and latency of disease. Eur J Clin Microbiol Infect Dis 13:908–914
92. Betts JC, Lukey PT, Robb LC et al (2002) Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. Mol Microbiol 43:717–731
93. Albrethsen J, Agner J, Piersma SR et al (2013) Proteomic profiling of *Mycobacterium tuberculosis* identifies nutrient-starvation-responsive toxin-antitoxin systems. Mol Cell Proteomics 12:1180–1191
94. Gopinath V, Raghunandanan S, Gomez RL et al (2015) Profiling the proteome of *Mycobacterium tuberculosis* during dormancy and reactivation. Mol Cell Proteomics 14:2160–2176
95. Schubert OT, Ludwig C, Kogadeeva M et al (2015) Absolute proteome composition and dynamics during dormancy and resuscitation of *Mycobacterium tuberculosis*. Cell Host Microbe 18:96–108
96. Li P, Seneviratne CJ, Alpi E et al (2015) Delicate metabolic control and coordinated stress response critically determine antifungal tolerance of *Candida albicans* biofilm persisters. Antimicrob Agents Chemother 59:6101–6112
97. Truong T, Zeng G, Lin Q et al (2016) Comparative ploidy proteomics of *Candida albicans* biofilms unraveled the role of the *AHP1* gene in the biofilm persistence against amphotericin B. Mol Cell Proteomics 15:3488–3500
98. Zhigang JS, Haoyue L, Jing C et al (2016) *Candida albicans* amphotericin B-tolerant persister formation is closely related to surface adhesion. Mycopathologia 181:41–49
99. Al-Dhaheri RS, Douglas LJ (2008) Absence of amphotericin B-tolerant persister cells in biofilms of some *Candida* species. Antimicrob Agents Chemother 52:1884–1887
100. Denega I, D'Enfert C, Bachellier-Bassi S (2019) *Candida albicans* biofilms are generally devoid of persister cells. Antimicrob Agents Chemother 63:e01979–e01918
101. Radzikowski JL, Vedelaar S, Siegel D et al (2016) Bacterial persistence is an active σ^S stress response to metabolic flux limitation. Mol Syst Biol 12:882
102. Balaban NQ (2004) Bacterial persistence as a phenotypic switch. Science 305:1622–1625
103. Gefen O, Gabay C, Mumcuoglu M et al (2008) Single-cell protein induction

dynamics reveals a period of vulnerability to antibiotics in persister bacteria. Proc Natl Acad Sci U S A 105:6145–6149

104. Vega NM, Allison KR, Khalil AS, Collins JJ (2012) Signaling-mediated bacterial persister formation. Nat Chem Biol 8:431–433
105. Wilmaerts D, Dewachter L, De Loose P-J et al (2019) HokB monomerization and membrane repolarization control persister awakening. Mol Cell 75:1031–1042
106. Jõers A, Kaldalu N, Tenson T (2010) The frequency of persisters in *Escherichia coli* reflects the kinetics of awakening from dormancy. J Bacteriol 192:3379–3384
107. Luidalepp H, Jõers A, Kaldalu N, Tenson T (2011) Age of inoculum strongly influences persister frequency and can mask effects of mutations implicated in altered persistence. J Bacteriol 193:3598–3605
108. Orman MA, Brynildsen MP (2013) Dormancy is not necessary or sufficient for bacterial persistence. Antimicrob Agents Chemother 57:3230–3239
109. Verstraeten N, Knapen WJ, Kint CI et al (2015) Obg and membrane depolarization are part of a microbial bet-hedging strategy that leads to antibiotic tolerance. Mol Cell 59:9–21
110. Henry TC, Brynildsen MP (2016) Development of Persister-FACSeq: a method to massively parallelize quantification of persister physiology and its heterogeneity. Sci Rep 6:25100
111. Sakakihara S, Araki S, Iino R, Noji H (2010) A single-molecule enzymatic assay in a directly accessible femtoliter droplet array. Lab Chip 10:3355–3362
112. Iino R, Matsumoto Y, Nishino K et al (2013) Design of a large-scale femtoliter droplet array for single-cell analysis of drug-tolerant and drug-resistant bacteria. Front Microbiol 4:300
113. Harms A, Fino C, Sørensen MA et al (2017) Prophages and growth dynamics confound experimental results with antibiotic-tolerant persister cells. mBio 8:e01964-17
114. Levin BR, Rozen DE (2006) Non-inherited antibiotic resistance. Nat Rev Microbiol 4:556–562
115. Ankomah P, Levin BR (2014) Exploring the collaboration between antibiotics and the immune response in the treatment of acute, self-limiting infections. Proc Natl Acad Sci U S A 111:8331–8338
116. Patra P, Klumpp S (2013) Population dynamics of bacterial persistence. PLoS One 8: e62814
117. Patra P, Klumpp S (2015) Emergence of phenotype switching through continuous and discontinuous evolutionary transitions. Phys Biol 12:046004
118. Gardner A, West SA, Griffin AS (2007) Is bacterial persistence a social trait? PLoS One 2:e752
119. Klapper I, Gilbert P, Ayati BP et al (2007) Senescence can explain microbial persistence. Microbiology 153:3623–3630
120. Levin-Reisman I, Brauner A, Ronin I, Balaban NQ (2019) Epistasis between antibiotic tolerance, persistence, and resistance mutations. Proc Natl Acad Sci 116:14734–14739
121. Feng J, Kessler DA, Ben-Jacob E, Levine H (2014) Growth feedback as a basis for persister bistability. Proc Natl Acad Sci U S A 111:544–549
122. Rotem E, Loinger A, Ronin I et al (2010) Regulation of phenotypic variability by a threshold-based mechanism underlies bacterial persistence. Proc Natl Acad Sci U S A 107:12541–12546
123. Lou C, Li Z, Ouyang Q (2008) A molecular model for persister in *E. coli*. J Theor Biol 255:205–209
124. Fasani RA, Savageau MA (2013) Molecular mechanisms of multiple toxin-antitoxin systems are coordinated to govern the persister phenotype Proc Natl Acad Sci U S A 110: E2528–E2537
125. Stepanyan K, Wenseleers T, Duéñez-Guzmán EA et al (2015) Fitness trade-offs explain low levels of persister cells in the opportunistic pathogen *Pseudomonas aeruginosa*. Mol Ecol 24:1572–1583
126. Nguyen D, Joshi-Datar A, Lepine F et al (2011) Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. Science 334:982–986
127. Dhar N, Mckinney JD (2010) Mycobacterium tuberculosis persistence mutants identified by screening in isoniazid-treated mice. Proc Natl Acad Sci U S A 107:12275–12280
128. Chua SL, Kuok J, Yam H et al (2016) Selective labelling and eradication of antibiotic-tolerant bacterial populations in *Pseudomonas aeruginosa* biofilms. Nat Commun 7:10750
129. Briers Y, Walmagh M, Van Puyenbroeck V et al (2014) Engineered endolysin-based "Artilysins" to combat multidrug-resistant gram-negative pathogens. mBio 5:e01379-14
130. Allison KR, Brynildsen MP, Collins JJ (2011) Metabolite-enabled eradication of bacterial persisters by aminoglycosides. Nature 473:216–220

131. Kwan BW, Chowdhury N, Wood TK (2015) Combatting bacterial infections by killing persister cells with mitomycin C. Environ Microbiol 17:4406–4414
132. Morones-Ramirez JR, Winkler JA, Spina CS, Collins JJ (2013) Silver enhances antibiotic activity against gram-negative bacteria. Sci Transl Med 5:190ra81
133. Starkey M, Lepine F, Maura D et al (2014) Identification of anti-virulence compounds that disrupt quorum-sensing regulated acute and persistent pathogenicity. PLoS Pathog 10:e1004321
134. Lebeaux D, Chauhan A, Létoffé S et al (2014) pH-mediated potentiation of aminoglycosides kills bacterial persisters and eradicates *in vivo* biofilms. J Infect Dis 210:1357–1366
135. Defraine V, Verstraete L, Van Bambeke F et al (2017) Antibacterial activity of 1-[(2,4-dichlorophenethyl)amino]-3-phenoxypropan-2-ol against antibiotic-resistant strains of diverse bacterial pathogens, biofilms and in pre-clinical infection models. Front Microbiol 8:2585
136. Stover CK, Warrener P, VanDevanter DR et al (2000) A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis. Nature 405:962–966

Part II

Quantification and Enrichment of Persister Cells

Chapter 2

Antibiotic Tolerance and Persistence Studied Throughout Bacterial Growth Phases

Enea Maffei, Cinzia Fino, and Alexander Harms

Abstract

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

Key words Antibiotic tolerance, Bacterial persistence, Bacterial resilience, Stationary phase, Growth media, Reproducibility, *Escherichia coli*, *Pseudomonas aeruginosa*

1 Introduction

Since their initial observation in the 1940s by Bigger, bacterial antibiotic tolerance and persistence have drawn the attention of researchers and clinicians all over the world [1]. By definition, antibiotic tolerance is the ability of bacteria to survive nominally lethal concentrations of bactericidal antibiotics, while antibiotic persistence denotes the survival of a typically nongrowing, antibiotic-tolerant subpopulation among a heterogeneous population of otherwise sensitive, growing cells [2]. Though these phe-

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nomena are thought to play major roles in antibiotic treatment failure and the frequent relapses of chronic infections [3, 4], their physiological basis and the underlying molecular mechanisms have remained largely unknown despite extensive research [5]. One major obstacle to progress in this field is that the published literature contains many contradictory or at least inconsistent findings [2, 5–8]. Dedicated research has suggested that these issues might be largely due to differences in the experimental methodologies used to assess antibiotic tolerance and persistence in the laboratory [2, 9–11]. In particular, the genetic background of the bacterial model organisms, their growth medium and culture conditions, and the inoculation method can have a large impact on the number of cells recovered after antibiotic treatment [9–13]. Recently, the field made an important effort to agree on common definitions of antibiotic tolerance and antibiotic persistence which included fundamental experimental guidelines on how to study these phenomena and how to distinguish them from antibiotic resistance [2]. However, no direct practical advice regarding experimental methodologies was included that could help researchers in the field understand their different results and guide newcomers around common pitfalls.

The methodology presented in this chapter was developed for *Escherichia coli* and *Pseudomonas aeruginosa*, two major model organisms in the field, and largely relies on the rigorous control of all relevant experimental variables by, for example, using a fully defined growth medium to avoid comparing "apples, oranges and unknown fruit" [14]. Rather than artificially inducing antibiotic tolerance through some kind of bacteriostatic treatment [15–17], our methodology solely focuses on antibiotic-tolerant cells that form in response to cues inherent to the bacterial life cycle such as starvation or entry into stationary phase. In addition, our methodology provides a complete view on the dynamics of antibiotic tolerance by recording the formation and disappearance of antibiotic-tolerant cells throughout the bacterial growth phases from lag after inoculation over exponential growth into early and late stationary phase. This comprehensive view enables the comparison of mutant strains with different growth dynamics and can reveal phenotypes that affect tolerance or persistence only in certain growth stages [8, 18]. Conversely, the focus on single, defined growth time points for antibiotic tolerance assays is prone to comparing bacteria in different growth stages and blind to whether observed differences between strains are, for example, due to changes in the formation or in the disappearance of antibiotic-tolerant cells.

2 Materials

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

1. 5× M9 salts solution: 33.9 g/L Na_2HPO_4, 15 g/L KH_2PO_4, 5 g/L NH_4Cl, 2.5 g/L NaCl. Dissolve the powder in ca. 80% of the final volume of the solution, for example, in 800 mL of water if you are preparing 1 L of solution, in a large beaker. Mix well using a magnetic stirrer. Once all the powder is dissolved, fill up to the desired volume using a graduated cylinder. M9 salts can either be prepared from the individual components (*see* **Note 3**) or be bought as premixed powder (e.g., Sigma-Aldrich M6030). This solution can be stored for years in the dark.
2. 100× trace elements solution: 0.18 g/L $ZnSO_4{\cdot}7H_2O$, 0.12 g/L $CuCl_2{\cdot}2H_2O$, 0.12 g/L $MnSO_4{\cdot}H_2O$, 0.18 g/L $CoCl_2{\cdot}6H_2O$. Weigh the powders into a beaker and dissolve directly into the final volume of water. Store in the dark.
3. 100 mM $FeCl_3$ solution: 16.22 g/L $FeCl_3$. Dissolve the powder directly into the final volume of water and mix well. Store in the dark at 4 °C. $FeCl_3$ is corrosive, so special care should be taken and gloves as well as protective goggles should always be worn when manipulating it.
4. 1 M $MgSO_4$ solution: 246.47 g/L $MgSO_4{\cdot}7H_2O$. Dissolve the powder in the final volume of water and mix well.
5. 1 M $CaCl_2$ solution: 147.01 g/L $CaCl_2{\cdot}2H_2O$. Dissolve the powder in the final volume of water and mix well.
6. 2.2 M or 40% (w/v) D-glucose solution: 440 g/L D-glucose monohydrate ($C_6H_{12}O_6{\cdot}H_2O$). Add the powder to ca. 70% of the final volume of water. Stir until everything is dissolved, subsequently fill up to the final volume using a measuring cylinder. Store at 4 °C (*see* **Note 4**).
7. M9Glc or M9-based culture medium (50 mL): 38.75 mL of ultrapure water (sterilized), 10 mL of 5× M9 salts solution, 500 μL of 40% w/v D-glucose solution, 500 μL of 100× trace elements solution, 30 μL of 100 mM $FeCl_3$ solution, 100 μL of 1 M $MgSO_4$ solution, 5 μL of 1 M $CaCl_2$ solution. Using sterile technique, mix all the substances in the order as listed to avoid precipitation. The final pH should be close to 7.2. Store at 4 °C for up to 1 week. Final molar concentrations of all

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

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

components are listed in Table 1 (*see* also **Note 5**). This medium is referred to as "M9Glc medium" throughout the chapter.

8. PBS (phosphate-buffered saline) solution: 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L $Na_2HPO_4{\cdot}2H_2O$, 0.24 g/L KH_2PO_4. Dissolve in 90% of the final volume of water and adjust the pH to 7.4 using 10 M NaOH. Fill up with water to the final volume and autoclave to sterilize.
9. LB agar plates: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L agar, square petri dishes (12 cm × 12 cm), round petri dishes (9.4 cm diameter). Mix all components and add water to the final volume. Sterilize by autoclaving. After sterilization, let the liquid LB agar cool down to ca. 60 °C and subsequently pour the required number of plates by dispensing 45 mL of LB agar per square petri dish or 24 mL of LB agar per round petri dish. Leave the agar to solidify with the lid closed at room temperature. After 2 days, the plates are sufficiently dry for usage and can be stored in tightly closed plastic bags at 4 °C for several weeks (*see* **Notes 6** and **7**).
10. Antibiotic stock solutions (50 mg/mL ampicillin; 1 mg/mL ciprofloxacin; 10 mg/mL tobramycin sulfate; 5 mg/mL meropenem trihydrate): Dissolve 53.14 mg ampicillin sodium salt; 1 mg ciprofloxacin; 10 mg tobramycin sulfate; or 5.7 mg meropenem trihydrate in 1 mL of ultrapure water each (*see* **Note**

8). Ampicillin sodium salt, ciprofloxacin, and tobramycin sulfate stocks can be prepared in advance and stored at −20 °C for several weeks, but multiple cycles of freezing and thawing should be avoided. Meropenem must be prepared freshly before each use due to low stability of the solution (*see* **Notes 8–10**).

11. Dilution plates: To prepare for serial dilutions, fill all wells in rows B-H of the required number of columns (one per sample) of a microtiter 96-well plate with 180 μL of PBS. To avoid evaporation, this should be done shortly before the serial dilutions are performed. Keep sterile.
12. Plastic test tubes: At least one sterile 1.5 mL test tube is needed per tested condition and time point. An excess of tubes should be sterilized by autoclaving before the experiment.
13. Flasks and glass tubes: One 50 mL Erlenmeyer flask per strain, one 100 mL Erlenmeyer flask per strain, and one cylindrical round-bottom glass culture tube with loose aluminium cap (15 mL nominal volume; 10 × 1.5 cm) per strain and condition are needed to determine levels of antibiotic-tolerant cells along the bacterial growth curve. One 50 mL Erlenmeyer flask per strain, one 500 mL Erlenmeyer flask per eight experimental conditions, and one cylindrical round-bottom glass culture tube with loose aluminium cap (15 mL nominal volume; 10 × 1.5 cm) per strain and condition are needed to perform the stationary-phase assay (*see* **Note 11**).
14. Graduated pipettes: To pipette and transfer larger volumes of media and culture, adequate sterile graduated pipettes should be used.
15. Bacterial strains: The protocols were developed for standard laboratory strains *E. coli* K-12 MG1655 F^- λ^- *ilvG*$^-$ *rfb-50 rph-1* (Coli Genetic Stock Center (CGSC) #6300) and *P. aeruginosa* PAO1 *Δpel Δpsl* [19] (*see* **Note 12**).
16. Cultivation and incubation: Bacterial liquid cultures should be agitated in a shaking incubator at 37 °C at 170 rpm. Agar plates should be incubated without agitation at 37 °C.

3 Methods

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

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

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

3.1 Determination of Antibiotic Tolerance Along the Growth Curve

3.1.1 Day 0: Collection of Materials and Inoculation of the Overnight Culture

1. Prepare or collect all the materials needed for the assay (**step 1** in Subheading 3.1.3 and following) (Table 2).
2. Pick an isolated bacterial colony and use it to inoculate 5 mL of M9Glc medium in a 50 mL Erlenmeyer flask. Incubate the preculture for 24 h at 37 °C shaking (170 rpm) (*see* **Notes 11–13**).

3.1.2 Day 1: Subculturing

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

3.1.3 Day 2: Antibiotic Tolerance Assay

1. Switch on the shaking incubator (37 °C, 170 rpm) at least 30 min before the start of the experiment to let it heat up. In the meantime, take the M9Glc medium out of the fridge, aliquot it into the glass flasks using sterile techniques, and let the medium reach room temperature. While waiting for the medium to warm up, the tubes needed for the assay can be labeled and other preparatory work can be done.
2. Dilute the preculture 1:100 into 10 mL of fresh M9Glc medium in a 100 mL Erlenmeyer flask and culture both the subculture and the preculture at 37 °C, 170 rpm. Repeat this subculturing step after 2 h and then six more times in a way that, eventually, eight cultures inoculated 8 h, 6 h, 5 h, 4 h, 3 h, 2 h, and 1 h before the last one are available (*see* **Notes 11, 12** and **14**).

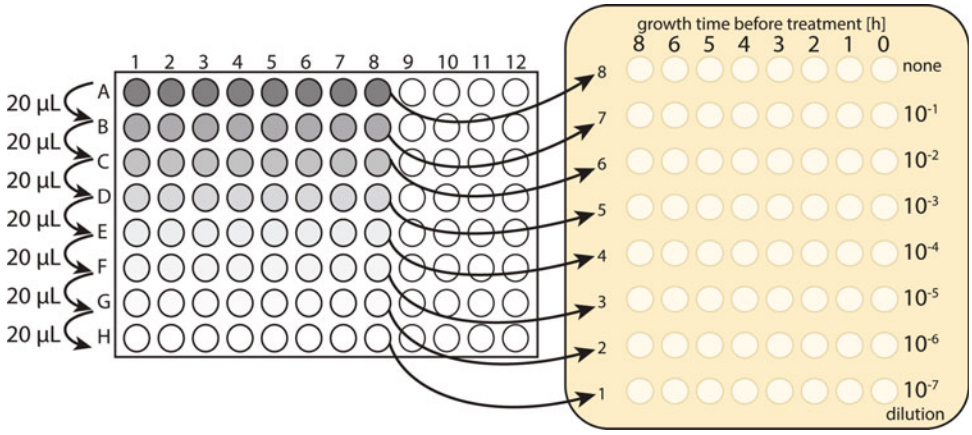

Fig. 1 Serial dilutions and spotting. This figure is a schematic representation of the serial dilutions performed in a 96-well microtiter plate (*left*) and the transfer of all dilutions onto a dry LB agar plate in a square petri dish for colony outgrowth (*right*). Briefly, 100 µL of each sample are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

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

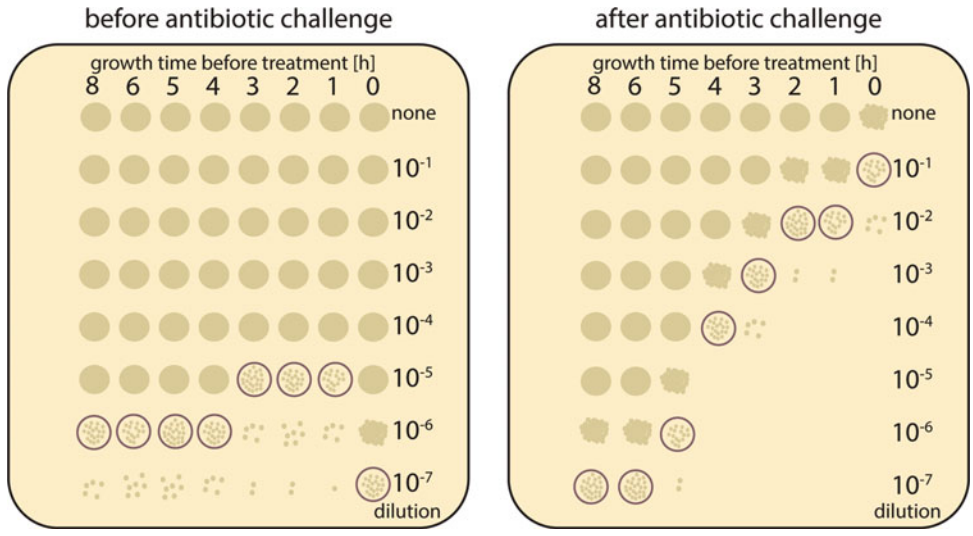

Fig. 2 Colony counts after CFU regrowth. This figure is a schematic representation of plates from a typical experiment of treatment of bacteria along the growth curve after colonies have grown for 24–48 h. Regions containing between 10 and 100 colonies are highlighted by a violet circle and should be used for CFU/mL calculation

8. After 5 h of antibiotic challenge, transfer 1.5 mL of each culture to a sterile 1.5 mL plastic tube and pellet the cells by centrifugation for 2 min at 18,000 × *g*. Remove the supernatant carefully and wash each pellet with 1 mL of sterile PBS. Start by washing the samples with the lowest incubation times and pay attention to the small, barely visible pellets that result from low-density cultures (*see* **Notes 17** and **18**). Repeat the centrifugation, remove the supernatant, and finally resuspend the pellets in 100 μL of sterile PBS.
9. Dilute and spot these samples obtained from the antibiotic-treated samples as described in Subheading 3.1.3 **steps 4** and **5** (Fig. 1).
10. Let the spots dry and incubate the plate at 37 °C for at least 16 h but monitor colony formation for 48 h (*see* Fig. 2 and **Notes 19** and **20**).

3.1.4 Day 3: CFU Counting

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

3.1.5 Day 4: CFU Counting and Plotting

1. Check the plates from day 3 and, if necessary, update the CFU counts. Subsequently, calculate the CFU/mL values for all samples before and after antibiotic treatment.

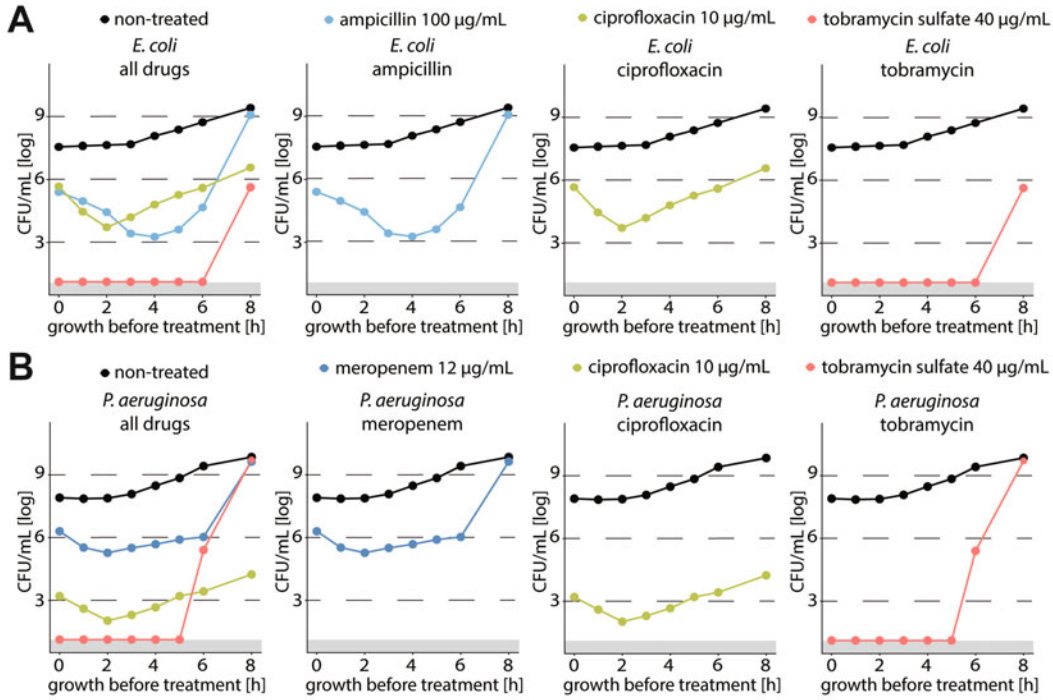

Fig. 3 Antibiotic tolerance of *E. coli* and *P. aeruginosa* along the growth phases. (**a**) Levels of *E. coli* K-12 MG1655 cells tolerant to 100 µg/mL ampicillin (light blue), 10 µg/mL ciprofloxacin (green), or 40 µg/mL tobramycin sulfate (red) were recorded throughout the bacterial growth phases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

2. Optional: Calculate the fraction of antibiotic-tolerant cells at each time point as the ratio of CFU/mL after and before antibiotic treatment.
3. To obtain independent biological replicates, the experiment should be performed at least three times on different days. When sufficient biological replicates have been performed, the data can be averaged and the standard error of the mean can be calculated. To calculate the average, transform all the CFU/mL values into their base 10 logarithm. Consequently, calculate the standard error of the mean on the individual log-transformed values.
4. Plot the recovered CFU/mL for each time point before and after antibiotic treatment on a base 10 logarithmic scale against time of growth before treatment (Fig. 3). If enough replicates have been performed, plot the mean values instead and include the respective error values as well. Note that these curves per se only report on antibiotic tolerance and that the inference of

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

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

antibiotic persistence requires additional time kill curves (*see* **Note 20**). The results and their interpretation are briefly discussed in **Note 21**.

3.2 Determination of Antibiotic Tolerance in Stationary Phase

3.2.1 Day 0: Preparation of Media and Cultures

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

3.2.2 Day 1: Subculturing

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

3.2.3 Day 3: Stationary-Phase Antibiotic Tolerance Assay

1. For the antibiotic treatment, transfer 5 mL of each culture from the Erlenmeyer flask into a glass tube and challenge with antibiotics at the desired final concentration. We typically treat with 100 μg/mL ampicillin (*E. coli*) or 12 μg/mL meropenem (*P. aeruginosa*), 10 μg/mL ciprofloxacin, and 40 μg/mL tobramycin sulfate. Place the tubes in a shaking incubator (37 °C/170 rpm) (*see* **Notes 11, 15** and **16**). Always include one untreated sample as a control for bacterial stationary-phase viability.
2. To determine viable cell counts, withdraw 200 μL aliquots from each glass tube at different time points, typically after 0 h (i.e., before addition of the antibiotics), 1 h, 3 h, 7 h,

24 h, as well as 48 h, and transfer into a 1.5 mL tube. Centrifuge each tube for 2 min at 18,000 × *g* and wash once with 200 μL of sterile PBS to remove excess antibiotic.

3. Transfer 100 μL of each culture into the wells of row A of a 96-well microtiter plate previously filled with 180 μL PBS. Perform 1:10 serial dilutions with the multichannel pipette by transferring 20 μL of the cultures from one row to the next containing sterile PBS. Mix well and change tips after every dilution (similar as shown in Fig. 1, left).
4. Use a multichannel pipet to transfer 10 μL spots of all dilutions from the 96-well plate onto a dry LB agar square petri dish (*see* **Note 6**; Fig. 1, right).
5. Wait for the spots to dry with open lid (e.g., next to a Bunsen burner or under a flow hood). Once the spots have dried, incubate the plate at 37 °C for 16–24 h.

3.2.4 Day 4: CFU Counting

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

3.2.5 Day 5: CFU Counting and Plotting

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

4 Notes

1. Sterilization by filtration should always be preferred since autoclaving can alter the chemical composition of solutions. This is particularly important for solutions containing complex molecules such as glucose which can degrade with heat [20].
2. All experimental steps, from the preparation of the media to the antibiotic killing assays, should be performed according to the rules of good laboratory practice [21].
3. If preparing the M9 salts solution from the individual components, be aware of water of hydration inside the salt crystals (e.g., disodium hydrogen phosphate is usually available as

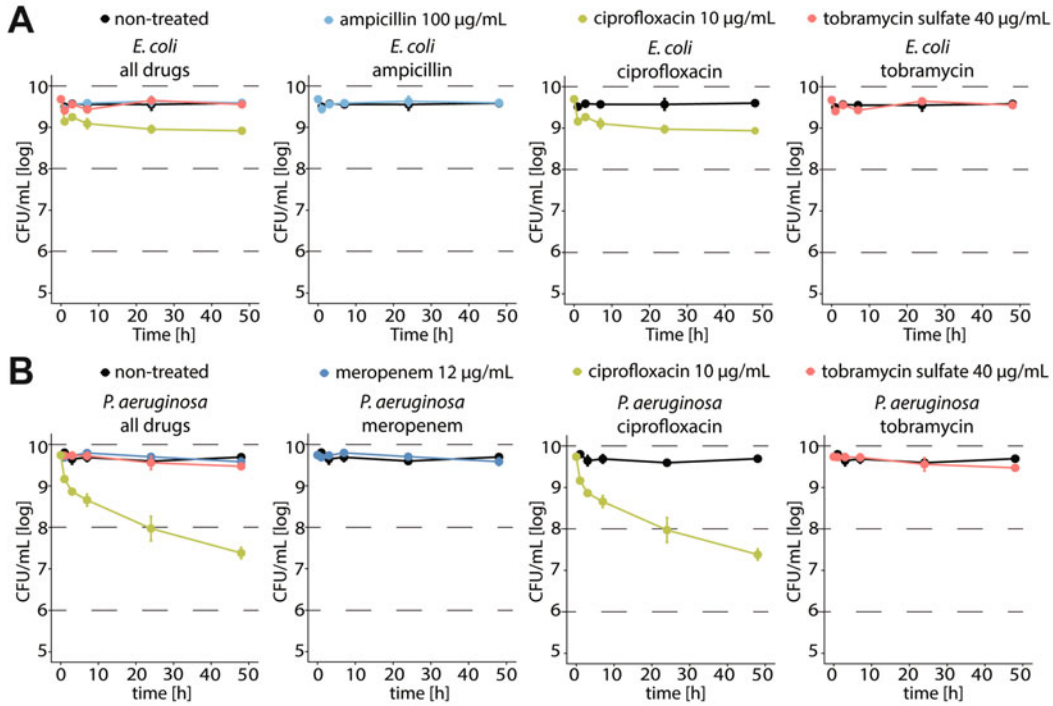

Fig. 4 Antibiotic persistence of *E. coli* and *P. aeruginosa* in stationary phase. (**a**) A stationary-phase culture of *E. coli* K-12 MG1655 was treated with 100 μg/mL ampicillin (light blue), 10 μg/mL ciprofloxacin (green), or 40 μg/mL tobramycin sulfate (red) and bacterial survival was recorded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

$Na_2HPO_4{\cdot}7H_2O$) and adapt the recipe accordingly to preserve molarities. The recipe described in this chapter corresponds to the quantities used by Sigma-Aldrich to prepare their variant of M9 salts powder (Sigma-Aldrich M6030). Note that there are some minor differences between M9 salts recipes that are circulating in the community, for example, between the recipe of Sigma-Aldrich and the one formulated by Cold Spring Harbor Protocols [22].

4. Dissolving the glucose may take up to 1 h. It is not recommendable to add water to dissolve the glucose powder more quickly, as the volume of the solution increases considerably when the D-glucose is dissolving. Instead, heating the solution gradually up to 40 °C can help dissolving the powder more quickly.
5. The main benefit of using a chemically defined medium prepared with ultrapure water is that the same cellular physiology can be obtained in every experiment and among different

laboratories. This is often not the case for complex media that may suffer from batch-to-batch variation due to poorly defined ingredients (e.g., tryptone and yeast extract) or due to degradation of some components if stored or prepared improperly [8, 12]. The trace element supplement is adapted from previous work by Gerosa et al [23]. Unless strictly necessary, we do not suggest to supplement the medium with thiamine (as it is done in many M9 medium recipes including the one of Gerosa et al [23]) in order to avoid that this compound might be used as an alternative carbon or nitrogen source. To assess antibiotic tolerance of thiamine auxotrophs with the present protocol, it is enough to supplement the medium with thiamine to a final concentration of 2.8 μM (as described in reference (23)). Any other auxotrophies can be complemented analogously.

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

but also other β-lactams can be used such as imipenem, piperacillin, or cefepime.

10. Antibiotic tolerance and persistence can either be confined to single drugs or affect multiple antibiotics, and the levels of antibiotic-tolerant cells can vary widely among different strains of the same species [25–27]. In order to obtain a complete picture, we therefore recommend to always separately assess tolerance to antibiotics with different modes of action such as β-lactams, fluoroquinolones, and aminoglycosides that are commonly used in the field (*see* also Figs. 3 and 4).
11. The proper aeration of a culture during long incubation times is essential to avoid uncontrolled changes of bacterial physiology that could affect outcome of the antibiotic tolerance assay. Therefore, make sure to cultivate the bacteria in a volume of medium that is around ten times less than the nominal fill volume of the Erlenmeyer flasks. Shaking tubes and flasks at 170 rpm is suitable to achieve both proper aeration and to prevent sedimentation of the bacteria. Whenever having a culture in a non–self-sustaining container (e.g., a glass culture tube), this should be inclined at ca. 45° to ensure optimal aeration. The inclination should be kept constant for the whole duration of the assay.
12. *P. aeruginosa* can form small aggregates in liquid batch culture that exhibit biofilm-like characteristics including, for example, increased antibiotic tolerance and therefore distort the results of antibiotic treatment assays. However, the formation of these aggregates can be largely avoided by using a mutant deficient in the production of Pel and Psl exopolysaccharides as well as by using precultures rather than direct inoculation from a single colony or cryostocks [9]. Nevertheless, visible aggregates of *P. aeruginosa* can transiently form during the precultures and subcultures but disperse again upon starvation [28]. Therefore, take care while pipetting to not include visible bacterial aggregates in the inocula for subcultures.
13. The colonies should be inoculated from a freshly streaked plate that is not older than 1 week. Alternatively, the preculture can also be inoculated directly from a cryostock. It is advisable to keep the inoculation method consistent among experiments, as it may have an influence on the results [9, 11].
14. It is recommended to prepare, collect, and label wherever necessary all the materials that are required throughout the steps prior to experimentation.
15. Measure the antibiotic minimum inhibitory concentration (MIC) by broth dilution assays in 96-well microtiter plates to make sure that the concentration used for the antibiotic

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

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

tolerance assay is considerably higher than this value [29]. If in doubt about the optimal antibiotic concentration for tolerance and persistence assays, test a range of concentrations and choose one that is so high that the killing rate does not increase anymore with increasing drug concentration. When comparing the antibiotic killing dynamics of different bacterial mutants or strains, measure the MIC of each of them. If they are very different, consider for your experiments that not only differences in antibiotic tolerance but also different intrinsic drug resistance (as expressed by MIC) can change the dynamics of bacterial killing in antibiotic treatment assays. *See* Table 4 for reference MIC values of *E. coli* K-12 MG1655 and *P. aeruginosa* PAO1.

16. Treating with 10 μg/mL ciprofloxacin, a very high concentration (ca. 1000× MIC), is necessary to avoid artefacts caused by secondary killing of antibiotic-tolerant bacteria due to the induction of prophages [8].
17. To increase accuracy when pipetting small pellets and to reduce the risk of disturbing the pellet, one can fit a regular 200 μL pipette tip on top of a 1000 μL tip. Like this, the large volume pipetted during washing can still be removed efficiently but the risk to wash out the small and sometimes invisible pellets at the bottom of the tube is reduced.
18. The pellets from samples of late-exponential-phase cultures treated with β-lactams are often difficult to homogeneously resuspend due to fulminant bacterial lysis and aggregation of cell debris. Vortexing the tubes for 15–30 s and pipetting the pellets up and down a few times are sufficient to reproducibly release the surviving bacteria into the PBS, because most of the hard pellets itself is merely composed of dead cell debris.

19. Bacteria treated with ciprofloxacin or tobramycin can take more than 36 h to form visible colonies on LB agar plates, in particular in case of *P. aeruginosa*. In order to include all bacterial survivors, it is therefore important to incubate the LB agar plates for 40–48 h in total at 37 °C before the final CFU counts are recorded.
20. The hallmark of antibiotic persistence is a time kill curve with a biphasic trajectory [2]. In cases where it is necessary to distinguish between antibiotic tolerance and persistence, it is therefore important to verify biphasic killing by determining viable CFU/mL not only after 5 h of drug treatment but also after, for example, 1 h, 3 h, and 7 h in order to record the dynamics of bacterial killing (like in a regular time kill curve). Previous work showed that bacterial killing under conditions similar to those presented in this protocol is largely biphasic [8, 18].
21. The results presented in Fig. 3 highlight a few important aspects. First, the overall shape of the tolerance curves along the growth phases are very similar for both *E. coli* K-12 MG1655 and *P. aeruginosa* PAO1 Δ*pel* Δ*psl*, suggesting that the underlying phenomena causing antibiotic tolerance are similar irrespective of the model organism investigated (similar to previous results [30]). Second, it is well visible how the initially higher tolerance levels (up to 2–3 h of cultivation) are a consequence of stationary phase carryover and drop as more bacteria exit lag phase [8]. Third, once bacteria start to successively enter stationary phase, the levels of tolerant cells increase again. For both organisms, tobramycin tolerance is only observed in stationary phase. The dynamics of ampicillin and ciprofloxacin tolerance are largely parallel except for the exponential phase of *E. coli* (ca. 3–6 h after subculturing) where the stark decrease in ampicillin-tolerant cells is not mirrored by the levels of ciprofloxacin-tolerant cells.
22. The results presented in Fig. 4 reveal a massive presence of antibiotic-tolerant cells for both *E. coli* K-12 MG1655 and *P. aeruginosa* PAO1 Δ*pel* Δ*psl* in the stationary phase. The biphasic kill curve resulting from ciprofloxacin treatment reveals a large fraction of persisters and at the same time indicates that most bacteria in culture are still active in DNA processing (as a prerequisite for gyrase poisoning by fluoroquinolones). Whereas the complete tobramycin tolerance of both organisms can be explained by reduced drug uptake due to lowered membrane potential, the total tolerance observed against β-lactams suggests absence of actively dividing cells in the later stages of stationary phase under these conditions [11, 31, 32].

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References

1. Bigger JW (1944) Treatment of staphylococcal infecctions with penicillin by intermittent sterilisation. Lancet 244:497–500
2. Balaban NQ, Helaine S, Lewis K et al (2019) Definitions and guidelines for research on antibiotic persistence. Nat Rev Microbiol 17:441–448
3. Mulcahy LR, Burns JL, Lory S, Lewis K (2010) Emergence of *Pseudomonas aeruginosa* strains producing high levels of persister cells in patients with cystic fibrosis. J Bacteriol 192:6191–6199
4. Lewis K, Manuse S (2019) Persister formation and antibiotic tolerance of chronic infections. In: Lewis K (ed) Persister cells and infectious disease. Springer, New York
5. Kaldalu N, Hauryliuk V, Tenson T (2016) Persisters-as elusive as ever. Appl Microbiol Biotechnol 100:6545–6553
6. Pontes MH, Groisman EA (2019) Slow growth determines nonheritable antibiotic resistance in *Salmonella enterica*. Sci Signal 12:eaax3938
7. Goormaghtigh F, Fraikin N, Putrinš M et al (2018) Reassessing the role of type II toxin-antitoxin systems in formation of *Escherichia coli* type II persister cells. mBio 9: e00640–e00618
8. Harms A, Fino C, Sørensen MA et al (2017) Prophages and growth dynamics confound experimental results with antibiotic-tolerant persister cells. mBio 8:1–18
9. Kragh KN, Alhede M, Rybtke M et al (2017) The inoculation method could impact the outcome of microbiological experiments. Appl Environ Microbiol 84:e02264–e02217
10. Heinemann M, Basan M, Sauer U (2020) Implications of initial physiological conditions for bacterial adaptation to changing environments. Mol Syst Biol 16:e9965
11. Luidalepp H, Jõers A, Kaldalu N, Tenson T (2011) Age of inoculum strongly influences persister frequency and can mask effects of mutations implicated in altered persistence. J Bacteriol 193:3598–3605
12. Sezonov G, Joseleau-Petit D, D'Ari R (2007) *Escherichia coli* physiology in Luria-Bertani broth. J Bacteriol 189:8746–8749
13. Goormaghtigh F, Van Melderen L (2016) Optimized method for measuring persistence in *Escherichia coli* with improved reproducibility. In: Michiels J, Fauvart M (eds) Bacterial persistence: methods and protocols. Springer, New York, NY
14. Neidhardt FC (2006) Apples, oranges and unknown fruit. Nat Rev Microbiol 4:876–876
15. Kwan BW, Valenta JA, Benedik MJ, Wood TK (2013) Arrested protein synthesis increases persister-like cell formation. Antimicrob Agents Chemother 57:1468–1473
16. Hong SH, Wang X, O'Connor HF et al (2012) Bacterial persistence increases as environmental fitness decreases. Microb Biotechnol 5:509–522
17. Vázquez-Laslop N, Lee H, Neyfakh AA (2006) Increased persistence in *Escherichia coli* caused by controlled expression of toxins or other unrelated proteins. J Bacteriol 188:3494–3497
18. Fino C, Vestergaard M, Ingmer H et al (2020) PasT of *Escherichia coli* sustains antibiotic tolerance and aerobic respiration as a bacterial homolog of mitochondrial Coq10. Microbiology 9:e1064
19. Broder UN, Jaeger T, Jenal U (2016) LadS is a calcium-responsive kinase that induces acute-to-chronic virulence switch in *Pseudomonas aeruginosa*. Nat Microbiol 2:1–11
20. Wang X-J, Hsiao K-C (1995) Sugar degradation during autoclaving: effects of duration and

solution volume on breakdown of glucose. Physiol Plant 94:415–418

21. Ridley R (2009) Handbook good laboratory practice (GLP): quality practices for regulated non-clinical research and development, special programme for research & training in tropical diseases (TDR), 2nd edn. WHO, Geneva
22. Cold Spring Harbor Protocols (2009) M9 salt solution. https://doi.org/10.1101/pdb.rec11973. Accessed 19 Oct 2020
23. Gerosa L, Kochanowski K, Heinemann M, Sauer U (2013) Dissecting specific and global transcriptional regulation of bacterial gene expression. Mol Syst Biol 9:658
24. Livermore DM (1995) Lactamases in laboratory and clinical resistance. Clin Microbiol Rev 8:557–584
25. Hofsteenge N, Van Nimwegen E, Silander OK (2013) Quantitative analysis of persister fractions suggests different mechanisms of formation among environmental isolates of *E. coli*. BMC Microbiol 13:25
26. Harms A, Maisonneuve E, Gerdes K (2016) Mechanisms of bacterial persistence during stress and antibiotic exposure. Science 354: aaf4268
27. Lewis K (2019) Persister cells and infectious disease. Springer, New York, NY
28. Schleheck D, Barraud N, Klebensberger J et al (2009) *Pseudomonas aeruginosa* PAO1 preferentially grows as aggregates in liquid batch cultures and disperses upon starvation. PLoS One 4:e5513
29. Wiegand I, Hilpert K, Hancock REW (2008) Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 3:163–175
30. Keren I, Kaldalu N, Spoering A et al (2004) Persister cells and tolerance to antimicrobials. FEMS Microbiol Lett 230:13–18
31. Damper PD, Epstein W (1981) Role of the membrane potential in bacterial resistance to aminoglycoside antibiotics. Antimicrob Agents Chemother 20:803–808
32. Tuomanen E, Cozens R, Tosch W et al (1986) The rate of killing of *Escherichia coli* by β-lactam antibiotics is strictly proportional to the rate of bacterial growth. J Gen Microbiol 132:1297–1304

Chapter 3

A Robust Method for Generating, Quantifying, and Testing Large Numbers of *Escherichia coli* Persisters

Silke R. Vedelaar, Jakub L. Radzikowski, and Matthias Heinemann

Abstract

Bacteria can exhibit phenotypes that render them tolerant against antibiotics. However, often only a few cells of a bacterial population show the so-called persister phenotype, which makes it difficult to study this health-threatening phenotype. We recently found that certain abrupt nutrient shifts generate *Escherichia coli* populations that consist almost entirely of antibiotic-tolerant cells. These nearly homogeneous persister cell populations enable assessment with population-averaging experimental methods, such as high-throughput methods. In this chapter, we provide a detailed protocol for generating a large fraction of tolerant cells using the nutrient-switch approach. Furthermore, we describe how to determine the fraction of cells that enter the tolerant state upon a sudden nutrient shift and we provide a new way to assess antibiotic tolerance using flow cytometry. We envision that these methods will facilitate research into the important and exciting phenotype of bacterial persister cells.

Key words Antibiotic tolerance, Flow cytometry, Nutrient shift, Tolerant cells, Persister cells, *Escherichia coli*

1 Introduction

Bacterial persistence is defined as the occurrence of cells within a population that are transiently tolerant against antibiotics without carrying a mutation conferring genetic resistance [1]. These antibiotic-tolerant cells have been suggested to be responsible for recurrent infections [2]. Tolerant cells can be formed stochastically in exponentially growing cultures [3]. Activation of toxin–antitoxin (TA) modules [2] and deletion of certain metabolic genes [4] has also been shown to increase the number of cells entering this

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phenotype. Certain environmental perturbations can also induce the fraction of tolerant cells in a population, such as the entry into stationary phase [5] or certain sudden nutrient shifts, in which case almost all cells in a population enter the tolerant state [6, 7].

The fact that certain nutrient shifts, for example, the one from glucose to fumarate in *E. coli* [8], can force almost all the cells in a population into the tolerant state offers new research opportunities to investigate the molecular basis of tolerance and persistence. While the investigation of the few stochastically occurring persisters in exponentially growing cultures and the very heterogeneous stationary-phase cultures require single-cell analyses or cell-sorting approaches, a population that consists almost entirely of tolerant cells allows the use of population-level high-throughput analyses. For example, by exploiting sudden nutrient shifts to generate almost homogeneous populations of tolerant cells, it was found that tolerant cells have increased ppGpp levels, are metabolically active with a metabolism geared toward energy generation and catabolism, and exhibit a proteome characterized by the σ^S-mediated stress response [8].

Despite the now enabled use of omics techniques for the study of bacterial persistence, experiments to assess how many cells are tolerant at which level of antibiotic exposure are still necessary. Here, in most cases, classical plating assays using different dilutions are performed to determine the colony-forming units (e.g., [5, 6]). However, this technique is very laborious and it has a high experiment-to-experiment variability requiring many plates to obtain statistically sound results [9]. Moreover, with plating fewer cells are recovered from their dormancy compared to recovery in liquid medium [10]. To this end, we recently introduced an alternative method to assess antibiotic tolerance with flow cytometry [8]. This method can be applied to populations solely consisting of dormant cells or to heterogeneous populations with dormant and growing cells. In this method, single cells' regrowth is assessed via temporal dilution of a fluorescent signal, which can originate from a stained membrane [11] or GFP expression [12].

In the current chapter we provide a detailed nutrient-shift-based protocol to generate large fractions of tolerant cells, which enables the study of tolerant cells on the population level. Furthermore, we illustrate a method to fluorescently stain the membrane of cells, which, together with flow cytometry and a Matlab script, allows to determine the fraction of cells that enter the tolerant state upon a sudden nutrient shift. Finally, we describe how the membrane-staining procedure together with flow cytometry can be used to assess tolerance.

2 Materials

Prepare all media using demi water.

2.1 Generation of Large Fractions of Tolerant Cells

1. LB agar plates: Prepare LB medium by adding tryptone (to a final concentration of 1% w/v), yeast extract (0.5% w/v) and sodium chloride (1% w/v) to a bottle with demi water. Add agar to a final concentration of 1.5% (w/v) and mix well. Autoclave for 15 min at 121 °C and let cool down to ~55 °C. Add antibiotics, if needed, and pour ~20 mL LB-agar per 10 cm petri dish (*see* **Note 1**). Store plates sealed with Parafilm at 4 °C with the agar side up. LB-plates containing antibiotics can be stored for up to 1 month.
2. M9 minimal medium: Prepare a base salt solution (211 mM Na_2HPO_4, 110 mM KH_2PO_4, 42.8 mM NaCl, 56.7 mM $(NH_4)_2SO_4$), a solution with trace elements (0.63 mM $ZnSO_4$, 0.7 mM $CuCl_2$, 0.71 mM $MnSO_4$, 0.76 mM $CoCl_2$), a solution with 0.1 M $CaCl_2$, one with 1 M $MgSO_4$, one with 1.4 mM thiamine-HCl, and one with 0.1 M $FeCl_3$. Autoclave the solutions with base salts, $CaCl_2$, $MgSO_4$, and trace elements, and store them at room temperature. Filter sterilize the thiamine and $FeCl_3$ solutions using a 0.22 μm PES filter (*see* **Note 2**) and store them at 4 °C. Stock solutions are sterilized so they can be stored for up to half a year. For 1 L M9 minimal medium, add 200 mL base salts, 700 mL water, 10 mL trace elements, 1 mL $CaCl_2$ solution, 1 mL $MgSO_4$ solution, 0.6 mL $FeCl_3$ solution and 2 mL thiamine solution. Fill up to 1 L with water and filter sterilize the resulting solution using a PES bottle top filter into an autoclaved 1 L bottle. Store at 4 °C for up to 1 month. The required carbon source is added just before the medium is used for cultivations.
3. 250 g/L glucose stock solution: For 100 mL, weigh 27.5 g D-glucose monohydrate and dissolve in 90 mL water. Adjust the pH to 7 using NaOH and fill up to 100 mL with water. Filter-sterilize the resulting solution using a PES bottle top filter into an autoclaved 100 mL bottle. Store at room temperature for up to 1 month.
4. 100 g/L fumarate stock solution: For 100 mL, weigh 14 g sodium fumarate and dissolve in 90 mL water. Adjust the pH to 7 using HCl and fill up to 100 mL with water. Filter-sterilize the resulting solution using a PES bottle top filter into an autoclaved 100 mL bottle. Store at room temperature for up to 1 month.
5. M9 minimal medium supplemented with glucose to a final concentration of 5 g/L for immediate use for cultivation:

Add 1 mL glucose stock solution (250 g/L) to 49 mL minimal medium in a 500 mL Erlenmeyer flask. Preheat and pre-aerate at 37 °C with shaking at 300 rpm before inoculation.

6. M9 minimal medium supplemented with fumarate to a final concentration of 2 g/L for immediate use for cultivation: Add 1 mL fumarate stock solution (100 g/L) to 49 mL minimal medium in a 500 mL Erlenmeyer flask. Preheat and pre-aerate at 37 °C with shaking at 300 rpm before inoculation.
7. Flow cytometer: Accuri C6, BD Biosciences or equivalent.

2.2 Identification and Quantification of Tolerant Cells Using Membrane Staining and Flow Cytometry

1. M9 minimal medium without carbon source, ice-cold.
2. M9 minimal medium with 1% (w/v) bovine serum albumin: Dissolve 0.5 g bovine serum albumin in 50 mL M9 minimal medium (room temperature) and filter sterilize the resulting solution using a PES bottle top filter into an autoclaved 100 mL bottle. Store at 4 °C.
3. Dye to stain cell membrane: PKH67 dye (Sigma), keep at 4 °C until use. Then dilute 50× in Diluent C, at room temperature.
4. Diluent C (Sigma, included in the PKH67 kit). Store at 4 °C, allow to warm to room temperature before the start of the staining.
5. M9 minimal medium without carbon source, at room temperature.
6. M9 minimal medium with fumarate added to a final concentration of 2 g/L, preheated to 37 °C and pre-aerated.

2.3 Assessment of Antibiotic Tolerance with Flow Cytometry

1. Antibiotics, prepare concentrated stocks of (*see* **Note 3**):
   (a) 100 mg/mL ampicillin: Dissolve 107 mg ampicillin sodium salt in 1 mL water. Filter-sterilize using a PES syringe filter and store at −20 °C.
   (b) 20 mg/mL tetracycline: Dissolve 108 mg tetracycline hydroxide in 5 mL water. Filter-sterilize using a PES syringe filter and aliquot stock in portions of 1 mL and store at −20 °C. Protect against light.
   (c) 50 mg/mL kanamycin: Dissolve 120 mg kanamycin sulfate in 2 mL water, filter-sterilize using a PES syringe filter, and store at −20 °C.
   (d) 35 mg/mL chloramphenicol: Dissolve 35 mg chloramphenicol in 1 mL 100% EtOH, filter-sterilize using a PES syringe filter and store at −20 °C.
   (e) 0.4 mg/mL trimethoprim: Dissolve 40 mg trimethoprim in 100 mL water. Filter-sterilize using a PES syringe filter and aliquot in portions of 1 mL and store at −20 °C.

(f) 1 mg/mL ofloxacin: Dissolve 10 mg ofloxacin in 10 mL water. Filter sterilize using a PES syringe filter and aliquot in portions of 1 mL and store at −20 °C.

(g) 1.25 mg/mL rifampicin: Dissolve 12.5 mg rifampicin in 10 mL water. Filter-sterilize using a PES syringe filter and aliquot in portions of 1 mL and store at −20 °C.

(h) 10 mg/mL carbonyl cyanide m-chlorophenylhydrazine (CCCP): Dissolve 20 mg CCCP in 2 mL pure methanol, filter-sterilize using a PES syringe filter and store at −20 °C.

2. LB medium: Add tryptone (to a final concentration of 1% w/v), yeast extract (0.5% w/v) and sodium chloride (1% w/v) to a final volume of 500 mL water. Autoclave for 15 min at 121 °C and sterilize using a 0.22 μm PES filter to remove debris, which disturbs the flow cytometric analyses (*see* **Note 4**). Store at room temperature.

3 Methods

3.1 Generation of Large Fractions of Tolerant Cells

To investigate tolerant cells at population level it is important to generate a cell population that consists almost entirely of dormant cells, even in the presence of a carbon source. In this section, we describe how to generate such a population by exposing the cells to a sudden nutrient shift. The procedure starts with generating an exponentially growing culture on glucose and continues with the steps to perform an abrupt switch to a different carbon source. In this section, fumarate is taken as the second carbon source because it generates the highest percentage of tolerant cells. Other gluconeogenic carbon sources can also be used for the switch [7]. A schematic overview of the method is shown in Fig. 1a–c.

1. Streak out an *E. coli* strain (e.g., BW25113) on LB-agar and incubate overnight at 37 °C. Full-grown plates can be stored up to one month at 4 °C sealed with parafilm.
2. Generate a culture that is fully exponentially growing on glucose, meaning that for several hours the cell number has been doubling at its maximal rate. Such a culture can be obtained by applying **steps 3–5** (*see* **Note 5**):
3. (Day 1) inoculate a 100 mL flask containing 10 mL M9 minimal medium supplemented with glucose to a concentration of 5 g/L with a single colony from an agar plate at around 5 p.m. Grow overnight at 37 °C and shaking at 300 rpm till around 9 a.m. the next morning.
4. (Day 2) to prepare the next preculture, first, determine the cell concentration in this overnight culture. For instance, dilute an

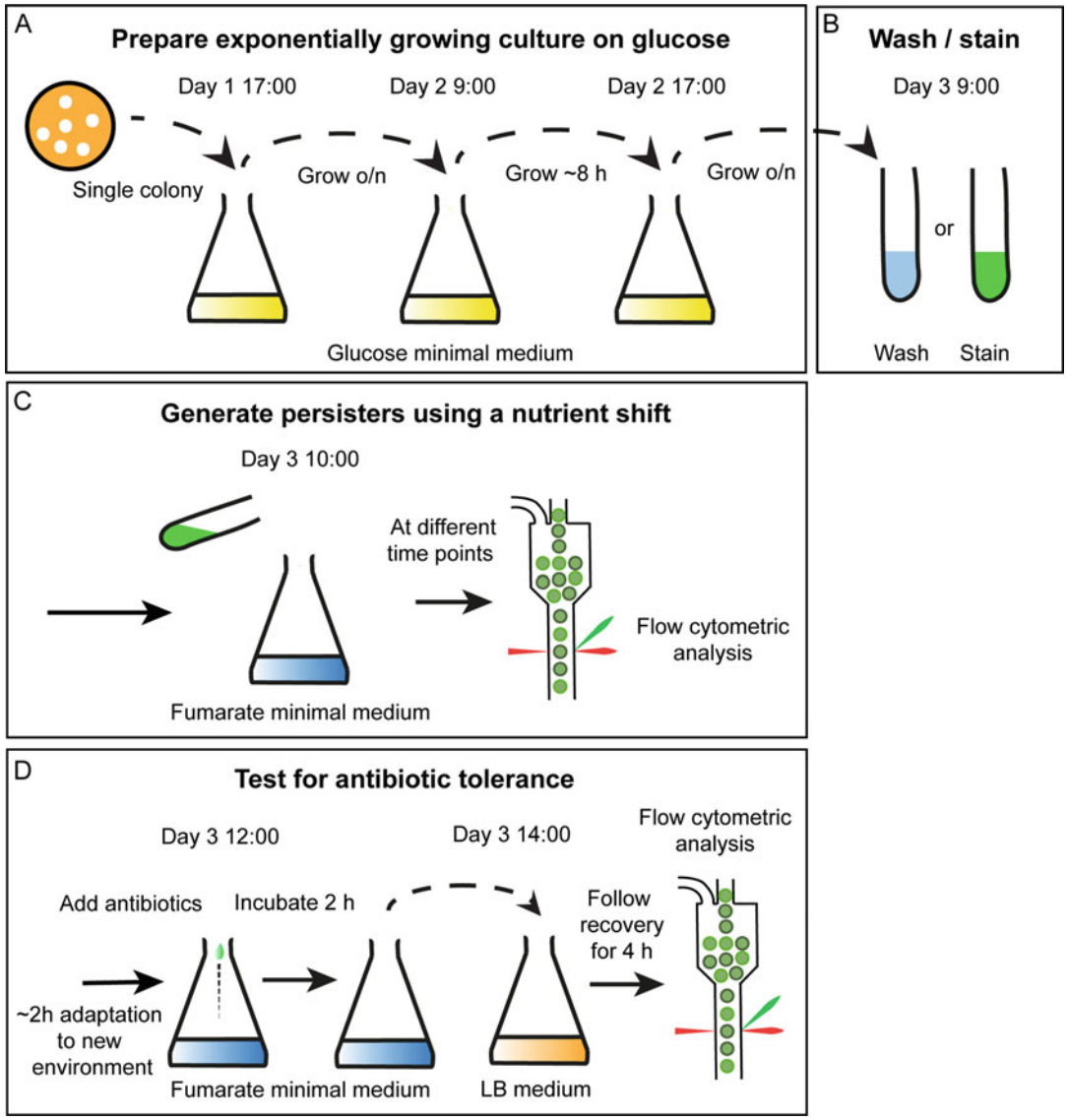

Fig. 1 Schematic overview of the procedure for generating persisters and for testing for their antibiotic tolerance. (**a**) Prepare an exponentially growing culture. Add a colony to 50 mL glucose minimal medium and grow overnight. Dilute this culture and grow during the day, dilute once more, and grow overnight. Make sure the culture is in exponential phase when starting the staining. (**b**) Wash or stain the cells to remove all residual glucose and optional, to stain the cells for tracking them with flow cytometry. (**c**) Add the washed or stained cells to fumarate minimal medium and follow their growth at different time points using flow cytometric analysis. (**d**) To test antibiotic tolerance, add antibiotics after 2 h in fumarate minimal medium. Incubate for 2 h and transfer 500 μL to 50 mL LB medium. Follow regrowth for 4 h using flow cytometry

aliquot of the culture 100×, transfer 200 μL to a 96-well plate (in case the used flow cytometer samples from 96-well plates) and measure the cell count (e.g., in 20 μL) with a flow

cytometer. Calculate the concentration in cells/mL, C, by using the following formula:

$$C = c \times d \times 50$$

where c is the number of cells counted in 20 μL and d is the dilution factor. Then determine the volume of culture to be added to a fresh flask for having a target cell density of 3×10^8 cells/mL at 5 p.m. on the same day as follows:

$$V_{\text{inoc}} = \frac{t_{\text{d}}}{2^{\frac{t}{\ln 2/\mu}}} \times \frac{C}{v}$$

where V_{inoc} is the volume that has to be added to the flask, t_{d} is the target density (in cells/mL) at the end of the day, μ is the growth rate of the exponentially growing population (in h^{-1}), t is the time until the end of the day (in h), and v is the culture volume (in mL). Add the calculated volume to a flask containing preheated (37 °C) M9 minimal medium with 5 g/L glucose, and grow the culture at 37 °C and shaking at 300 rpm.

5. (Day 2) to perform the actual nutrient shift the next morning (day 3) with a culture that is fully exponentially growing on glucose, perform another subculturing step the evening before (on day 2) to have 3×10^8 cells/mL at the desired time point in the morning of day 3. To calculate the volume that has to be added to a new flask, repeat **step 4**. Because of the huge dilution that has to be made for the inoculation of this overnight culture, it is recommended to inoculate several parallel flasks with dilutions such that one surely obtains at least one culture at the right cell density (*see* **Note 6**). Also, to prevent lag phase behavior, which can mess up the timing, it is recommended to use preheated and pre-aerated medium.
6. (Day 3) to start the nutrient shift, wait until the culture has reached OD_{600} of 0.3–0.8 before continuing with the nutrient shift. When the culture reaches the desired OD_{600}, measure the cell concentration of the culture with the flow cytometer. The cell concentration should be around $2–5 \times 10^8$ cells/mL. With the knowledge of the cell concentration, calculate the volume of the culture that contains 1.5×10^9 cells.

   When staining is applied, skip the next steps and go immediately to Subheading 3.2.
7. Wash the cells to remove any residual glucose. Therefore:
8. Transfer the calculated volume to a 15 mL falcon tube and spin down the culture for 5 min at $3000 \times g$, 4 °C, and discard the supernatant.
9. Wash the pellet with 5 mL ice-cold M9 minimal medium without carbon source by carefully pipetting. The ice-cold medium is used to slow down the cells' metabolism. Spin

down, remove supernatant, and wash once more with the same volume of M9 minimal medium.

10. Spin down the culture for 5 min at 3000 × *g*; 4 °C, discard the supernatant and resuspend the pellet in 1 mL M9 minimal medium without carbon source.
11. Transfer the cells to a 500 mL flask containing 50 mL pre-heated M9 minimal medium with 2 g/L fumarate. The cell concentration in the new flask is important because the initial cell density influences the fraction of cells that enter the tolerant state after the nutrient shift (*see* **Note 7**). With *E. coli* BW25113, this procedure results in about 99.99% of cells entering the tolerant state [7, 8]. With other *E. coli* strains, this fraction can be different.

3.2 Identification and Quantification of Tolerant Cells Using Membrane Staining and Flow Cytometry

To determine the fraction of cells that enter the tolerant state, for example, after the nutrient shift, one can stain the membrane of the cells with a fluorescent dye, follow the fluorescence of the cell population after the nutrient shift over time and analyze the resulting single-cell fluorescence data with a computational script. The dye we propose (PKH67, Sigma) stains the membranes of the cells and equally distributes over the two daughter cells after cell division. Thus, the fluorescence intensity of a cell halves with every division. Knowing the initial fluorescence intensity of the population after staining and comparing this with the fluorescence intensity of a cell at a later time point, one can calculate how often a cell has divided [7]. Specifically, to estimate the fraction of cells that have not started to divide, a Matlab script is used that fits a mathematical model to the time course data (i.e., cell counts, fluorescence intensity distributions). The model assumes that the fluorescence intensity of a cell decreases by half with each cell division and that cells have some autofluorescence. Further, it assumes exponential growth of the growing cells from some time point after the nutrient shift onward. As the fluorescence intensities of individual cells are not identical, the model fits bimodal distributions to the fluorescence intensity distributions determined at the different time points and estimates the growth rates of the two populations of cells based on the total cell counts determined and fitted bimodal distributions. The model, its rational, and mathematics are explained in detail in our previous paper [7].

To determine the fraction of dormant cells that emerge after a sudden nutrient shift and to estimate the growth parameters (including, among others, the growth rates of both populations), membrane staining is applied in combination with the nutrient shift method to generate tolerant cells, meaning that the washing **steps 7–11** in Subheading 3.1 are replaced by the following staining protocol. A schematic overview of this method is shown in Fig. 1a–c.

1. Generate exponentially growing cells on glucose according to **steps 1–6** of Subheading 3.1.
2. Before the shift to fumarate, cells are stained with a fluorescent dye. Before the staining, take Diluent C from the PKH67 kit from the fridge and allow it to warm to room temperature. The dye should stay in the fridge.
3. Determine the volume of the glucose culture that contains 1.5×10^9 cells. Transfer the calculated volume to a 15 mL falcon tube. Spin down the culture for 5 min at 3000 × *g*; 4 °C (Eppendorf centrifuge). Discard the supernatant very carefully by pipetting (*see* **Note 8**).
4. During the centrifugation mentioned in the previous step, prepare a mix of 500 μL of room-temperature Diluent C with 10 μL of the dye solution (*see* **Note 9**).
5. When performing **steps 5–9** act fast and respect the timings (*see* **Note 10**). Resuspend the cell pellet in 500 μL of solely Diluent C (room temperature) by carefully pipetting up and down. Make sure that the pellet is fully dissolved and that there are no droplets on the sides of the tube.
6. Add the prepared dye solution (10 μL of dye in 500 μL of Diluent C at room temperature) to the cell solution and mix briefly by vortexing. Incubate the cells for exactly 3 min at room temperature (*see* **Note 11**).
7. Immediately after 3 min add 4 mL of ice-cold M9 medium with 1% (w/v) BSA and mix briefly by vortexing (*see* **Note 12**).
8. Centrifuge the cells for 5 min at 3000 × *g*; 4 °C. Discard the supernatant by pipetting and resuspend the cells in 5 mL of ice-cold M9 without carbon source and centrifuge the cells again (5 min; 3000 × *g*; 4 °C).
9. Wash the cells once more in the same manner. Resuspend the cells in 1 mL M9 medium without carbon source at room-temperature and transfer them to the preheated M9 minimal medium with 2 g/L fumarate (*see* **Note 13**) to yield an OD_{600} of approximately 0.6, which corresponds to a cell concentration of ~5×10^8 cells/mL. Check the quality of the staining using flow cytometry by measuring a proper dilution of the culture. Well-executed staining should have only one peak on the FL1 (533 nm) signal, and the cells should be 100-fold brighter than unstained cells.
10. To obtain data that can be used with the Matlab script, the measurements must be done at specific times. The first time point must be gathered as soon as possible after the switch.
11. The next data time point should be one in which the growing population is becoming visible as a separate peak in the FL1 histogram. This time point varies depending on the conditions and carbon sources used (*see* **Note 14**).

12. After taking these first time points, it is advisable to take measurements every 30–60 min, depending on the growth rate of the growing population, until the growing population count is at least equal, or higher than the non-growing population count.

3.3 Guide on How to Use the Matlab Script

The Matlab script and exemplary input files are provided on GitHub (https://github.com/molecular-systems-biology). The following steps describe the data acquisition and data format requirements.

1. To assess tolerant cells, it is important to generate tolerant cells as described in Subheading 3.1 and to stain them as described in Subheading 3.2. The staining needs to be of an appropriate quality for the script to work optimally (*see* **Note 15**).
2. The data needs to be gathered over the growth of the culture according to **steps 10–12** in Subheading 3.2 (*see* **Note 16**). Certain factors need to be considered when gathering the data, for it to be usable with the script. The input data needs to be provided as a CSV file containing the flow cytometry measurements for each cell at each time point, without a header (File 1). In the Matlab script, the following input needs to be provided.
   (a) The number identifying the column of the CSV file containing the relevant fluorescence intensity (FI) data (in case of our example data as provided at GitHub, the number is 3—variable SC).
   (b) The scaling factor. Different flow cytometers have different sensitivity and numerical output values. This factor is used to accommodate for these differences and make sure that the data from different machines can be used within the script capabilities (variable maxval_new_FC).
   (c) The times when the samples were taken (variable tt).
   (d) The absolute cell concentration in the culture at the respective time points (variable cc).
   (e) The number of cells (or rows) in each CSV data file (variable g).
   (f) A specification of which of the data files should be used for the fitting. Depending on the nutrient switch, sometimes it takes hours before the growing population is numerous enough to be detected. The first time point used for the fit should have this population visible. Some data points can be excluded, for example, if the measurement has failed or has been inaccurate for any scientifically justified reason (variables indx and cc_indx).

Table 1
Overview of the parameters needed to be set for Matlab script. Left column: overview of parameters that need to be set. Right column: instructions on how to estimate these values

Parameter	How to determine
Non-growing population initial FI mean	From "Fluorescence Data" figure—*see* Fig. 2a
Non-growing population FI stdev	From "Fluorescence Data" figure—*see* Fig. 2a
Non-growing population cell concentration	The cell concentration after the nutrient switch is usually a good initial guess, except for nutrient switches in which the non-growing population is small. Upper and lower bounds can be very relaxed
Growth rate of non-growing population	From previous experiments
Growing population initial FI mean	From "Fluorescence Data" figure—*see* Fig. 2a
Growing population FI stdev	From "Fluorescence Data" figure—*see* Fig. 2a
Initial growing population cell concentration	A guesstimate based on the expected number of adapting cells for a particular carbon source switch. Upper and lower bounds can be very relaxed
Growth rate of the population that starts to grow normally	From previous experiments determining growth rate on particular carbon source
Unstained cell background autofluorescence	By analyzing data for unstained cells, or by checking the FI for very late samples, when cells have lost all their fluorescence

In the Matlab script, one needs to define the allowed ranges of the parameters to be estimated as well as initial parameter guesses. These values should be close to the true values. Table 1 provides an overview of the parameters that need to be set and instructions on how to estimate these values, for example, by visual inspection of the data or by using previous knowledge. It is advisable to run the script once on the data before setting the parameters. From the then generated "Fluorescence Data" figure (Fig. 2a), one can identify initial guesses (in matrix variable IG) and ranges for some of the parameters (in matrix variable bounds).

1. Two figures generated by the script are crucial at determining the quality of the fit, that is, the figure "Cellcount curve fit check" and the multipanel figure "Bi-gaussian fit for each time point." An example of a good fit is shown in Fig. 2; two examples of bad fits are shown in the Supplementary Figs. 1 and 2. The files for these fits are provided with the Matlab script. The two respectively generated figures show the cell count and fluorescence intensity data along with the plotted model predictions for both the growing and non-growing populations, and the sum of these populations. The way the model prediction fits the data can be used to assess whether the parameters are estimated correctly.

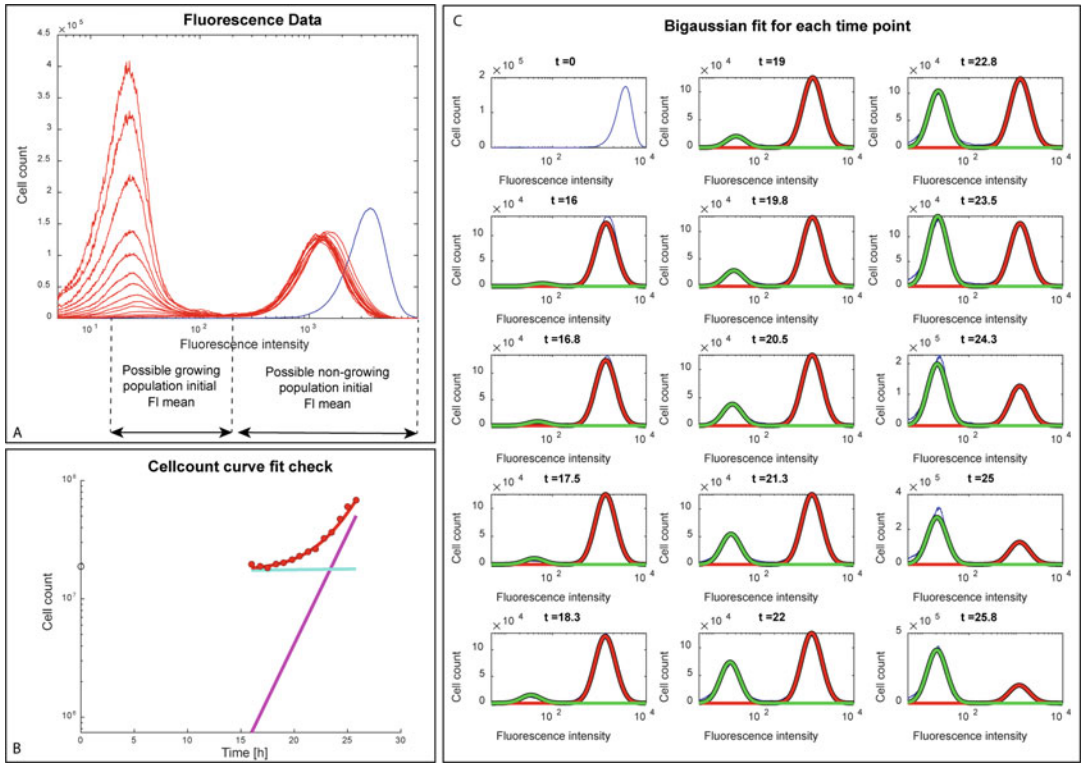

Fig. 2 Finding initial parameters and example of a good fit. (**a**) How to find the initial parameter guesses using the Fluorescence Data figure generated by the script. The model needs a good estimation input to generate proper estimations. Therefore, the mean fluorescence of the growing and the non-growing population needs to be estimated. To make a good estimation pick the average of the fluorescence in between the left arrows for growing cells and the average of the peak on the right for non-growing cells. (**b**) The cell count curve fit check. Empty disk—cell count at $t = 0$, red disks—cell counts used for the model fit, red line—predicted total cell count, cyan line—cell count of non-growing cells, magenta line—cell count of growing cells. (**c**) The model fit to the fluorescence data at each time point. Blue line—experimental data, green line—the distribution corresponding to the growing population, red line—the distribution corresponding to the non-growing population, black line (not visible in this specific case)—the sum of the distributions pictured by red and green line

2. In the first bad fit (Supplementary Fig. 1), the parameter bounds are set wrong. The FI means for both populations are overestimated, and the bounds are outside of the correct values. Moreover, the nongrowing population cell concentration is underestimated.
3. From the plots (Supplementary Fig. 1a), certain problems can be deduced.
   (a) The red line on the left panel that describes the modeled cell counts does not appear to fit with the cell count points from measurements. This is caused by the underestimation of the non-growing population cell concentration.

(b) The green line and the red line on the right panel do not fit the bimodal distribution. This is caused, on top of cause from problem 1, by the overestimation of the FI means.

4. Moreover, we can see from the text output of the script that some of the parameters—the growth rate of the growing population in this case—reaches the lower limit of the bounds set, and thus it is not estimated correctly. However, we know what the growth rate of the used strain in given conditions should be and the fact that the estimate is equal to one of the boundaries is just a symptom of another issue, and not the issue itself.
5. If we fix the first problem and correct the nongrowing population cell concentration to a value that is close to the measured value, we obtain graphs shown in Supplementary Fig. 2. While the graph on the left looks like it is a good fit, the graph on the right has the similar problems as before, stemming from the overestimation of the FI means. Fixing these values as described above, we can obtain a good fit.
6. It is clear from the plots shown in Fig. 2b, c that the model closely fits the data and the data output can be trusted.
7. The parameters obtained from the fitting are output as text. The Matlab script used as an example generates the following data.

```
alpha = 0.00047173
mu_growin = 0.43374
mu_nongrw = 0.0026477
sgma_grow = 53.0783
sgma_nong = 55.9828
I_0 = 3419.6016
EI = 20.0569
dye_degr = 0.015
BG cutoff = 1
BG magnit = 1
BG width = 1
CC weight = 1
Bigaus pt = 16.0833 16.8333 17.5833 18.3333 19.0833 19.8333
20.5833 21.3333 22.0833 22.8333 23.5833 24.3333 25.0833
25.8333
CC pts = 16.0833 16.8333 17.5833 18.3333 19.0833 19.8333
20.5833 21.3333 22.0833 22.8333 23.5833 24.3333 25.0833
25.8333
```

8. The most important obtained values are alpha (fraction of cells that entered dormancy after the nutrient shift), mu_growin (growth rate of the growing population), mu_nongrw (growth rate of the nongrowing population). All these and other parameters, except alpha, should be checked against the lower and

upper bounds set in the script and if they are equal to them, the bounds should be relaxed until they do not limit the results anymore.

3.4 Assessment of Antibiotic Tolerance with Flow Cytometry

The ability to regrow after antibiotic treatment is an indication that a cell has been in the tolerant state during treatment. While typically such regrow experiments are done with plating assays (i.e., determination of colony-forming units), here we proposed a method to perform such regrow assessments with flow cytometry. These assays require the above mentioned membrane staining. Despite the staining procedure that needs to be carried out, this technique is less laborious and has less variability than plating assays [9]. Also, it was found that more cells were able to wake up after dormancy when liquid medium is used compared to regrowth on plates [10]. A schematic overview of this method is shown in Fig. 1d.

1. To assess the antibiotic tolerance of cells, it is important to generate tolerant cells as described in Subheading 3.1 and to stain them as described in Subheading 3.2.
2. After transfer to the fumarate medium, it takes a certain time for the non-adapting cells to develop full tolerance against antibiotics. It is recommended to let the cells adapt for at least 2 h after staining (*see* **Note 17**) before treating them with antibiotics.
3. After >2 h, add the antibiotics. We tested 100 μg/ml ampicillin, 20 μg/mL tetracycline, 100 μg/mL kanamycin, 140 μg/ml chloramphenicol, 5 μg/mL trimethoprim, 100 μg/ml rifampicin, 5 μg/ml ofloxacin, and 50 μg/ml CCCP [8]. Concentrations were obtained from literature and from survival assays on cells growing on glucose (*see* **Note 18**).

   Because some cells undergo a reductive division after the switch to fumarate, the number of cells after 2 h is slightly higher than at time point zero. Therefore, it is required to measure a dilution of the culture directly after staining and right before antibiotics are added.
4. Incubate the cultures that now contain the antibiotics for 2 h whilst shaking at 300 rpm at 37 °C. After the incubation time, measure again a dilution of the culture with the flow cytometer to determine the cell count. The cell count should not have increased after the addition of antibiotics. In some cases, the cell count can even be decreased, for example, when a bacteriolytic antibiotic is used (*see* **Note 19**).
5. To assess the fraction of cells being able to recover from antibiotic treatment, the antibiotics are diluted out. Pipet 500 μL of the culture into 50 mL preheated and filtered LB medium (*see* **Note 14**), mix well and transfer a sample of the non-diluted culture in the 96-well plate (from where the flow cytometer will

take the sample) and measure it straight away with the flow cytometer (*see* **Note 20**). The first sample taken from the LB culture is very important because it reflects all cells in their dormant state and it is used to calculate the fraction of cells that became dormant after the nutrient switch (*see* **Note 21**).

6. From now on, subject the LB cultures to flow cytometric analyses every 30 min for a period of 4 h. Cells have a short doubling time on LB and thus cell counts can quickly enter a range that is beyond the linear range of the flow cytometer. Take notice of the cell count in each sample and determine the appropriate dilution for the next sample (*see* **Note 22**).
7. The fraction of tolerant cells is calculated by subtracting the number of non-dividing cells (cells that do not exit the bottom right part of Fig. 3a) in each time point from the initial number of nondividing cells.

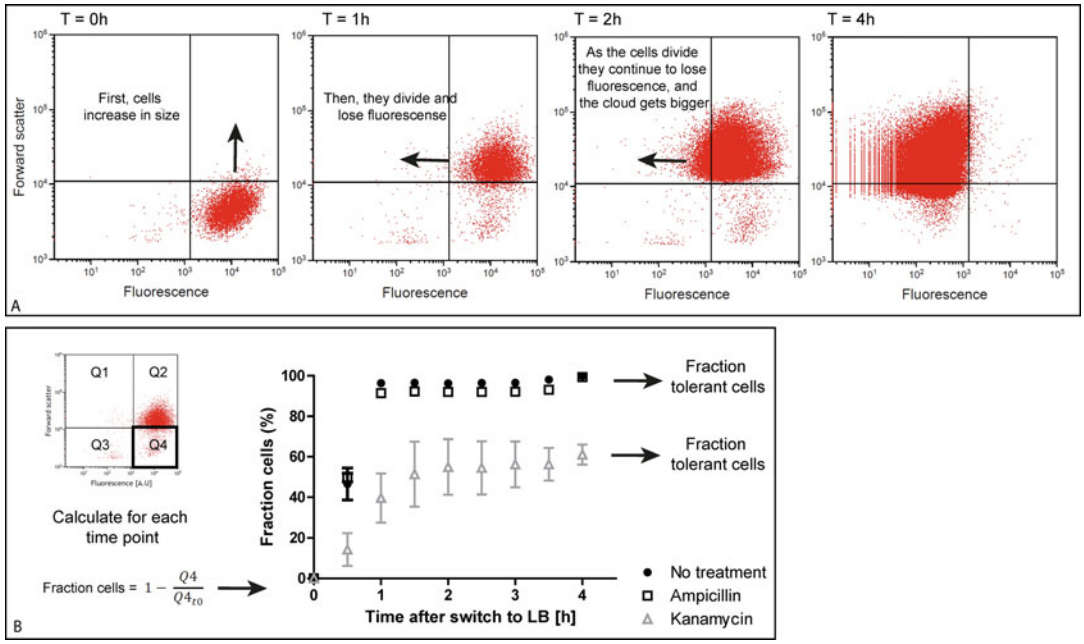

Fig. 3 Example of regrowth in LB medium after antibiotic treatment. (**a**) Exemplary flow cytometry graphs over time. When tolerant cells (stained with fluorescent dye) are transferred to LB, they first increase in size followed by a loss in fluorescence as a consequence of their divisions. Cells in Q1 are big and have lost their fluorescence. Cells in Q2 are big and have a high fluorescence intensity. Cells in Q3 are small and have lost their fluorescence. Cells in Q4 are small and are fluorescent. (**b**) Left, the formula of how the fraction of tolerant cells is calculated. Right, an example graph of treatment with two different antibiotics. The fraction of cells for each time point is calculated by 1 minus the number of cells in Q4 divided of the number of cells in Q4 on time point 0. Cells that are killed by antibiotics will not regrow in LB medium and therefore will not leave section Q4 in the flow cytometer graph. After 4 h, a steady state is reached and the fraction of cells on $t = 4$ can therefore be used as the ultimate fraction of viable cells

8. Samples measured directly after staining, after the addition of antibiotics, and right before the nutrient shift are solely used as quality controls for the experiment. The cells should show one single small peak directly after the switch to fumarate and the population should have significantly slowed-down growth before antibiotics are added. After the 2 h treatment with antibiotics the growth should have completely stagnated and the cell number can even drop in case a bacteriolytic antibiotic (e.g., ampicillin) is used.
9. For data analysis of the total fraction of tolerant cells, the cells are followed for 4 h after the switch to LB, and the loss of fluorescence as well as the size of the cells is used to determine the fraction of the population being able to escape dormancy after antibiotic treatment. It is required to set up a dot plot to gate the cells (FSC-H vs SSC-H) and a dot plot using cell size and fluorescence (FL1-A vs FSC-A).
10. To determine the fraction of cells escaping dormancy, the number of cells transferred to LB is taken at time point zero (T0). For each following time point, the number of nondividing cells is subtracted from the cells transferred at T0. It is not possible to use the number of escaping cells since they start dividing after the switch to LB (*see* **Note 23**) as shown in Fig. 3a.
11. To define the non-growing population, the quadrant function, as in Fig. 3a, needs to be adjusted manually for each sample. Especially in samples where a big part of the population just started to divide, it can be hard to distinguish the non-growing population from the growing population. It is advised to use the later time points to set the quadrant and use the same settings for the early division samples (*see* **Note 24**). When one is only interested in the final number of tolerant cells and not in the recovery dynamics, one can decide to not analyze these samples. An example of the recovery dynamics is shown in Fig. 3b.
12. The data from 4 h on LB is used to calculate the final fraction of tolerant cells. Take the number of nondividing cells and subtract it from the number of cells added to LB at T0. It is optional to take the average of the last two, or the last three measurements to calculate the final fraction of tolerant cells (only if the recovered fraction has reached steady state). Averages and standard deviations are calculated from three individual experiments and shown as box plots.

4 Notes

1. If antibiotics are required it is important that the antibiotic is always added after autoclaving the LB-agar and after the LB agar has cooled down to ~55 °C, to avoid degradation of the antibiotic. It is recommended to prepare 1000× concentrated stocks of the required antibiotics, to store them at −20 °C and thaw them just before pouring plates.
2. When using the glucose-to-fumarate rapid nutrient shift to generate large fractions of tolerant cells, it is important to not use cellulose acetate filters because these filters could release acetate into the medium [13], which *E. coli* can use as a carbon source. As *E. coli* can have different preferences on which gluconeogenic carbon source to use first, even a small concentration of acetate could affect the results. PES filters are equally priced and do not release any compound that can act as a substrate for *E. coli*.
3. The antibiotic concentrations used in the working solutions are based on literature research and on experiments where we tested the antibiotics on *E. coli* cells growing on fumarate. The concentration of the antibiotic stocks is based on the solubility of the antibiotic. For ampicillin and tetracycline, the solubility is very high so it was decided to make a 1000× concentrated stock.
4. When measuring and counting *E. coli* cells, the flow cytometer needs to be calibrated for small cells. Typically, the default settings are not suitable for *E. coli* cells. With the BD Accuri C6, the threshold for *E. coli* should be 8000 for the forward scatter (FSC-H) and 500 for the side scatter (SSC-H). Further, debris in the medium can disturb the measurement. Thus, it is required to only use filtered medium when measuring samples with the flow cytometer. For reliable cell counts with the Accuri C6 flow cytometer, the events measured by the flow cytometer should be between 10,000 and 100,000 cells in 20 μL of the measured sample. This level ensures a sufficiently high number of cells in comparison to machine noise, and is sufficiently low to not cause over-saturation of the instrument. For LB it is important to autoclave and filter sterilize the medium before use in the flow cytometer. LB contains, when only autoclaved, a lot of components that lead to a strong background signal in the flow cytometer. This interferes with the sample because the debris appears around the same size as your cells on the FSC-SSC dot plot and therefore it is required to use filtered LB medium to lower the background signal.
5. For the successful generation of almost 100% tolerant cells with a glucose-to-fumarate shift, the cells in the glucose culture must be in a fully exponentially growing state on glucose for

at least 24 h before the nutrient shift. Because cells typically have a lag phase after inoculation from the LB plate, we recommend to start culturing cells at least 1 day before the actual nutrient shift to ensure the maximal growth rate on glucose.

6. To ensure that cells keep on growing exponentially also after the re-inoculation, it is crucial to pre-heat the medium before dilution of the cultures. Even then, typically short lag phases occur after diluting, and it is therefore recommended to not only inoculate the calculated number of cells but also to prepare a preculture starting with 2× the number of cells required. In so doing, at the desired time point in the morning one surely obtains a culture with the proper cell density.
7. Previous research has shown that the initial cell density after the nutrient shift influences the fraction of dormant cells after a nutrient shift [14]. The absolute number of adapting cells seems to be constant regardless of the initial cell density after a glucose-to-fumarate shift, resulting in a higher fraction of cells adapting when a flask is inoculated with a low cell density [13]. Furthermore, note that with different *E. coli* wild-type strains, different fractions of dormant cells can emerge.
8. Do not lose any cells when applying the staining, because the dye-to-cell number ratio is very important. Losing cells will increase the dye-to-cell ratio causing cells to be stained too intensely, which in turn can lead to cell death. A suboptimal dye-to-cell number ratio can also affect the viability of the cells, resulting in fewer cells being able to recover after antibiotic treatment.
9. Preparing this mixture too soon will cause the dye to clump and staining intensity will be suboptimal. The solution can be prepared during the first centrifugation step, although the Diluent C must be brought to room temperature earlier. When multiple samples are stained in parallel, always change the pipette tip to prevent water transfer to the vial containing the dye stock. The dye-to-cell number ratio is very important for proper staining. The ratio is good when the cells clump a bit during staining, as indicated by a slightly cloudy solution.
10. Leaving droplets on the side of the tube will create a fraction of unstained cells because droplets containing cells do not get in touch with the dye. This can be seen as a separate unstained fraction and will influence results or even make them unusable.
11. Longer incubation will cause the cells to die, whereas shorter incubation will cause the cells to be stained suboptimally. If you stain cells of multiple different samples in parallel, add the dye in 20–30 s intervals, such that the incubation time can be strictly adhered to.

12. BSA blocks the remaining dye molecules, thereby preventing the cells from being killed. It is recommended to use a timer and make sure you have the M9 medium with BSA in your pipette ready so that you only have to release it from the pipette into the tube containing the cells when the 3 min incubation time is over.
13. It is recommended to prepare the flasks with preheated and pre-aerated M9 medium with 2 g/L fumarate before harvesting the cells.
14. The higher the fraction of adapting cells, the sooner the first usable data point can be taken. In the case of a glucose-to-fumarate switch, 15–16 h after the switch is usually a good starting point, as the growing population starts to be large enough to be detectable, while it still has a fluorescence intensity that is above the autofluorescence. Capturing time points in which the growing population still has some fluorescence above the cellular autofluorescence is crucial for the script to estimate the fraction of growing cells accurately.
15. To obtain a good fit of the data to the model and thus reliable parameter estimates, the data needs to fulfill certain criteria. First, the fluorescence intensity of stained cells should be at least two orders of magnitude higher than the background fluorescence of unstained cells. This, in turn, means that the fluorescence intensity decrease can be tracked over 5–6 divisions, which is enough for most carbon source switches. Moreover, the fluorescence intensity data of stained cells should form a single peak in the histogram. The narrower the peak, the better, as the growing and nongrowing populations will be better separated from each other in the obtained data. While in our experiments we had the best results using the green fluorescent dye, we have also used a red fluorescent dye (PKH26), which has giving us data of lesser quality. In principle, any product that utilizes a fluorophore linked to an aliphatic chain that intercalates in the cell membrane could be utilized to generate the data for the script, and there are many alternative products available on the market that have different excitation and emission properties that might be best suitable for the equipment used. However, due to different protocols and properties of these dyes, appropriate controls would have to be made to exclude the effect of the staining procedure on the obtained results.
16. The fluorescence intensity data from the flow cytometer needs to be in log10 space. Depending on the cytometer used, a transformation needs to be done on the data (as we do in case of the Accuri C6 cytometer) or is already done in the cytometer or its software. Moreover, depending on the flow cytometer, the numerical range of values can be different and this needs to be addressed by setting the scaling factor.

17. After transfer to the fumarate medium, several cells will undergo a reductive cell division within the first hour. Experiments have shown that after 2 h on fumarate, all cells of the population become tolerant against ampicillin [8]. Adding antibiotics too early might result in fewer cells surviving treatment, whereas incubating longer than 2 h does not increase the size of the tolerant population.
18. For each antibiotic used in our experiments, we checked in the literature which concentrations were used in *E. coli* inhibition experiments. We used this information as a starting point to explore which concentration of each antibiotic kills growing cells. Before the tolerance experiments were done we carried out identical experiments with glucose-grown cells to determine the concentration of antibiotics needed to kill a growing population.
19. When switching the cells to fumarate, 0.01% of the population will adapt and start proliferating [7]. Those cells will be sensitive to antibiotic treatment. Especially when the tolerant cells are kept for longer periods (~24 h after the nutrient shift), a significant population of growing cells is visible which are sensitive to antibiotics. When a bacteriolytic antibiotic such as ampicillin is used, the treatment will cause these cells to lyse. Therefore, the cell count after treatment can be lower than before treatment.
20. The sample should not be diluted because there are now 100 times less cells in the culture than in the flask where the cells were incubated with the antibiotics.
21. To check the cells' ability to survive antibiotic treatment they are transferred to LB medium. No washing steps are applied to protect the cells from extra stress. By only transferring 500 μL in 50 mL the antibiotics are diluted 100-fold, enough to nullify their inhibiting effect.
22. Bacteria grow fast on LB medium, $\mu = 1.9\ h^{-1}$ [15]. Therefore, to generate reliable data points it is essential to take regular measurements. Dead cells do not proliferate, meaning that their number remains the same and they do not lose their fluorescence. They are visible as a stained cloud of small cells in the FL1-A versus forward scatter (FSC-A) plot. However, when the descendants of the small growing population keep increasing their number, they outgrow the linear range of the flow cytometer and a bigger dilution must be made to measure the sample. This has the risk to lose the visibility of the population of dead cells because they are excessively diluted and their cell count is not reliable. In particular, when only a small fraction of cells are not dividing, a small dilution introduces a big measurement error by diluting out the number of non-growing cells.

23. Bacteria escaping the dormant state do this by resuming growth. Since it is impossible to determine the individual growth rate for each cell, there is no way to use the growing population for calculating the fraction of cells able to escape dormancy. However, since we know the original number of cells transferred to LB and we can distinguish growing from nondividing cells, we can use the number of nondividing cells and the number of cells at T0 to calculate the fraction of the population which has survived antibiotic treatment.
24. When cells start waking up from their dormant state, they will first get bigger, followed by the loss of fluorescence. This can be seen in a dot plot as in Fig. 3a (FL1-A vs FSC-A). However, in some cases, the cells may wake up a bit slowly, and in the earlier time points of the recovery experiment, the population of cells with increasing size might overlap with the nondividing population. In that case, it is advised to determine the nondividing population at a later time point and use those settings in the more indefinite sample.

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References

1. Balaban NQ, Helaine S, Lewis K et al (2019) Definitions and guidelines for research on antibiotic persistence. Nat Rev Microbiol 17:441–448
2. Lewis K (2010) Persister cells. Annu Rev Microbiol 64:357–372
3. Feng J, Kessler DA, Ben-Jacob E, Levine H (2014) Growth feedback as a basis for persister bistability. Proc Natl Acad Sci U S A 111:544–549
4. Moyed HS, Bertrand KP (1983) *hipA*, a newly recognized gene of *Escherichia coli* K-12 that affects frequency of persistence after inhibition of murein synthesis. J Bacteriol 155:768–775
5. Nguyen D, Joshi-Datar A, Lepine F et al (2011) Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. Science 334:982–986
6. Amato S, Brynildsen M (2015) Persister heterogeneity arising from a single metabolic stress. Curr Biol 25:2090–2098
7. Kotte O, Volkmer B, Radzikowski JL, Heinemann M (2014) Phenotypic bistability in *Escherichia coli*'s central carbon metabolism. Mol Syst Biol 10:736
8. Radzikowski JL, Vedelaar S, Siegel D et al (2016) Bacterial persistence is an active σ^S stress response to metabolic flux limitation. Mol Syst Biol 12:882
9. Jett BD, Hatter KL, Huycke MM, Gilmore MS (1997) Simplified agar plate method for quantifying viable bacteria. BioTechniques 23:648–650
10. Wong FH, Cai Y, Leck H et al (2020) Determining the development of persisters in extensively drug-resistant *Acinetobacter baumannii* upon exposure to polymyxin B-based antibiotic combinations using flow cytometry. Antimicrob Agents Chemother 64:e01712–e01719
11. Rousselle C, Barbier M, Comte V et al (2001) Innocuousness and intracellular distribution of PKH67: a fluorescent probe for cell

proliferation assessment. In Vitro Cell Dev Biol Anim 37:646–655

12. Jõers A, Liske E, Tenson T (2020) Dividing subpopulation of *Escherichia coli* in stationary phase. Res Microbiol 71:153–157
13. Vos KD, Burris FO, Riley RL (1966) Kinetic study of the hydrolysis of cellulose acetate in the pH range of 2–10. J Appl Polym Sci 10:825–832
14. Radzikowski J (2012) Systems biology of bacterial persistence, a metabolism-driven strategy for survival. University of Groningen, Dissertation, Groningen
15. Schmidt A, Kochanowski K, Vedelaar S et al (2016) The quantitative and condition-dependent *Escherichia coli* proteome. Nat Biotechnol 34:104–110

Chapter 4

Enrichment of Persister Cells Through B-Lactam-Induced Filamentation and Size Separation

Etthel Windels, Bram Van den Bergh, and Jan Michiels

Abstract

Analyzing persisters at the single-cell level is crucial to properly define their phenotypic traits. However, single-cell analyses are challenging due to the rare and temporary nature of persister cells, thus requiring their rapid and efficient enrichment in a culture. Existing methods to isolate persisters from a bacterial population show important shortcomings, including contamination with susceptible cells and/or cell debris, which complicate subsequent microscopic analyses. We here describe a protocol to enrich persisters in a culture using β-lactam-induced filamentation followed by size separation. This protocol minimizes the amount of cell debris in the final sample, facilitating single-cell studies of persister cells.

Key words Persister, Enrichment, Single-cell

1 Introduction

As bacterial persistence involves phenotypic heterogeneity within a population, interrogation of the persister physiology requires the selective enrichment of persister cells in a population. Many standard microbiological and molecular techniques run into limitations at this point, as persisters are often present at very low frequencies and existing fluorescent markers do not reliably distinguish persisters from susceptible cells, resulting in samples that are highly contaminated with susceptible cells [1–3]. Persisters can be enriched in a culture through prolonged antibiotic treatment that kills susceptible cells, as antibiotic tolerance is the only feature that is currently known to characterize all persister cells [4, 5]. However, the resulting sample is dominated by dead cell debris and is therefore inappropriate for microscopic studies. Using an antibiotic that lyses susceptible cells, followed by sedimentation of intact cells, only slightly reduces contamination by cell debris [6]. In addition to low final persister densities, the above-described methods involve prolonged antibiotic exposure, which potentially affects

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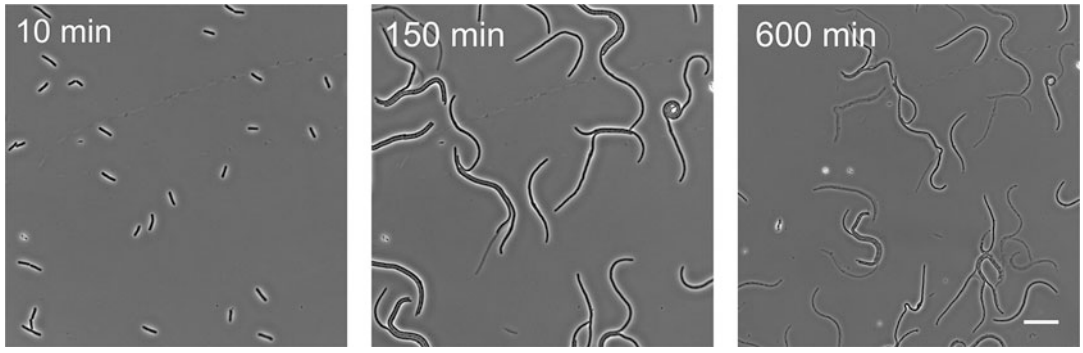

Fig. 1 Microscopy images of a culture treated with cephalexin. During treatment with 50μg/ml cephalexin, susceptible exponential-phase cells filament severely, followed by cell lysis (scale bar: 20μm). Performing size separation at maximal cell length, but before lysis is initiated, allows for efficient enrichment of antibiotic-tolerant cells (Reproduced from [10])

persister formation [7–9]. We here propose a persister enrichment method that overcomes these technical hurdles. This method exploits the unique killing properties of cephalexin, a β-lactam antibiotic that first induces strong filamentation before lysis is initiated (Fig. 1). Enriching antibiotic-tolerant persisters by size separation right before lysis of susceptible cells occurs, limits the antibiotic exposure time and results in a sample containing a high density of persister cells and very little cell debris, making it particularly useful for subsequent (single-cell) microscopic studies [10].

2 Materials

Prepare all media using deionized water. Store at room temperature, unless indicated otherwise.

1. Antibiotic stock solution (1000×): Weigh 50 mg of cephalexin 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 (*see* **Note 1**). Filter-sterilize (0.22μm) and store aliquots immediately at −20 °C.
2. Mueller Hinton Broth (MHB): Follow the instructions of the supplier of premixed MHB powder (*see* **Note 2**).
3. 10 mM $MgSO_4$ solution: Weigh 2.46 g of $MgSO_4 \cdot 7H_2O$ and add water to a final volume of 1 L before autoclaving.
4. Sterile microcentrifuge tubes (1.5 ml).
5. Sterile plastic tubes (50 ml), suitable for centrifugation.
6. Sterile glass test tubes and Erlenmeyer flasks (250 ml).
7. Sterile Erlenmeyer filtration flasks (500 ml) with side arm.

8. Büchner funnel, including a supporting adapter cone that fits the filtration flasks.
9. Polyvinylidene fluoride membrane filters with a diameter corresponding to the Büchner funnel diameter and a pore size of 5μm (*see* **Note 3**).
10. Vacuum pump with connection tubing that fits the filtration flasks.
11. Microcentrifuge capable of 4000 × *g*.
12. Centrifuge for 50 ml tubes capable of 4000 × *g*.
13. Incubator at 37 °C, capable of orbital shaking at 200 rpm.

3 Methods

Work at room temperature and under sterile conditions. Incubation is carried out at 37 °C, shaking at 200 rpm. A schematic overview of the method is provided in Fig. 2 (*see* **Note 4**).

3.1 Installation of the Vacuum Filtration System

1. Connect the vacuum pump to the side arm of a sterile filtration flask.
2. Place the Büchner funnel with adapter onto the filtration flask and make sure the flask is sealed airtight.
3. Place a membrane filter wetted with sterile growth medium in the Büchner funnel.

3.2 Persister Enrichment Protocol

1. Inoculate the strain under investigation in a test tube containing 5 ml MHB and incubate for 20 h.

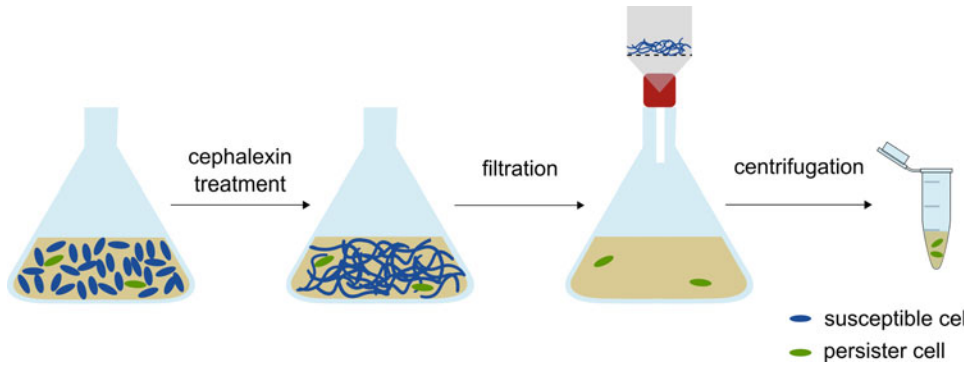

Fig. 2 Schematic overview of persister enrichment protocol. A bacterial culture of $\pm 10^6$ CFU/ml is treated with 50μg/ml cephalexin for 60 min. During treatment, susceptible cells strongly filament but do not yet lyse, while persisters are not affected by the antibiotic. The treated culture is vacuum filtered (pore size of 5μm) to separate short, antibiotic-tolerant persisters from filamented, susceptible cells. After filtration, the culture is centrifuged to wash away the antibiotic and to increase the density of the resulting sample (Reproduced from [10])

2. Dilute the overnight culture 1:5000 in 100 ml MHB and incubate for 90 min (*see* **Note 5**). When working with *Escherichia coli*, a cell density of $\pm 10^6$ CFU/ml should be attained at this point (*see* **Notes 6** and **7**).
3. Treat the culture with 50μg/ml cephalexin for 60 min (*see* **Notes 8–10**). During treatment, antibiotic-susceptible cells should strongly filament but not yet lyse (Fig. 1).
4. Pour the culture in the Büchner funnel and perform vacuum filtration (*see* **Note 11**).
5. Replace the filtration flask containing the filtered culture with an empty, sterile flask. Replace the used membrane filter with a fresh filter.
6. Pour the filtered culture in the Büchner funnel and perform vacuum filtration.
7. Collect the filtrate in sterile 50 ml tubes.
8. Centrifuge the tubes at 4000 × *g* for 5 min. Pour off the supernatant.
9. Resuspend the pellet in the remaining volume. Transfer this volume to a microcentrifuge tube. Pooling cells from different tubes into one microcentrifuge tube increases the cell density in the final sample.
10. Centrifuge the microcentrifuge tubes at 4000 × *g*. Pipette off the supernatant and resuspend in 500μl 10 mM $MgSO_4$ by vortexing.
11. Centrifuge the microcentrifuge tubes again at 4000 × *g*. Pipette off the supernatant and resuspend in a small volume of 10 mM $MgSO_4$ or MHB, depending on the subsequent experiment. For microscopy experiments, resuspend the pellet in a few μl of 10 mM $MgSO_4$ or MHB by pipetting up and down. A representative example of a resulting sample is shown in Fig. 3.

4 Notes

1. Add drops of 1 M NaOH to increase the solubility of cephalexin in deionized water.
2. Using premixed MHB formulas may enhance reproducibility. 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. Store the autoclaved medium at room temperature and away from light. Do not store the medium for longer than a week after autoclaving. Never autoclave the medium twice.

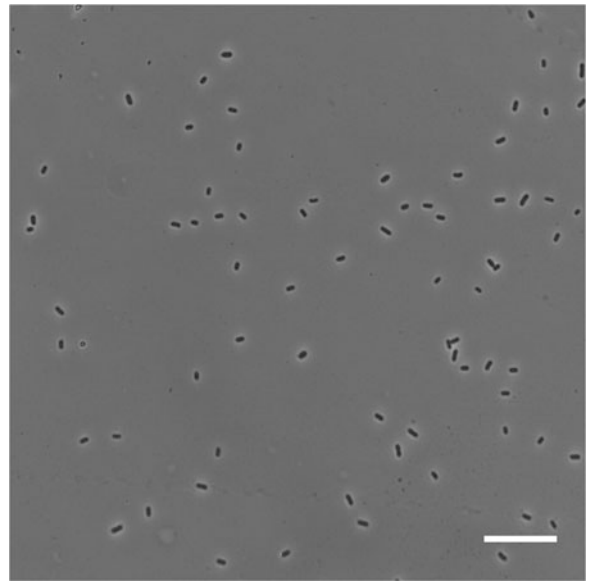

Fig. 3 Microscopy image of a sample after cephalexin treatment and vacuum filtration. The final sample contains antibiotic-tolerant cells that did not filament in response to cephalexin treatment (scale bar: 20 μm). As filtration is performed before susceptible cells start lysing, the sample contains a very limited amount of cell debris (Reproduced from [10])

3. Filters with a pore size of 5μm are convenient for *E. coli*, as normal *E. coli* cells have an average cell length of 2μm while cephalexin-treated cells elongate to 10–100μm. Adjust the pore size according to the organism under study and the filamentation effect of the used antibiotic.
4. The protocol described in this chapter is validated for *E. coli* K-12 MG1655 in MHB medium with cephalexin treatment. Optimization of the protocol is advisable when using other strains, media or antibiotics (*see* **Notes 6**–**8** and **10**).
5. Depending on the desired number of persisters in the final sample, a larger culture volume can be used.
6. When using other strains or species, take into account that the efficacy of cephalexin is highly dependent on the cell density at the onset of treatment. The overnight culture should be sufficiently diluted to ensure a low cell density when treatment is initiated ($\pm 10^6$ CFU/ml, although this depends on the treatment concentration of cephalexin). For *E. coli* K-12 MG1655, stronger dilution results in less persisters in the final sample, while weaker dilution results in less efficient cephalexin-induced elongation.
7. When using other strains or species, take into account that the incubation time after dilution and before treatment should be sufficiently long in order for susceptible cells to escape the lag

phase. However, incubating the culture for too long results in a too high cell density at the onset of treatment (*see* **Note 6**). An optimal combination of the dilution factor and the incubation time before treatment should be found in order to achieve an efficient cephalexin treatment.

8. Cephalexin can be replaced by another antibiotic with similar, filamentation-inducing effects (e.g., ciprofloxacin or aztreonam). For an efficient size separation, it is important that the size of filamented cells sufficiently differs from nonfilamented cells.
9. For *E. coli* K-12 MG1655, a cephalexin treatment concentration of 50μg/ml corresponds to 6.25× the minimum inhibitory concentration.
10. When using another strain or antibiotic, the treatment concentration and treatment duration should be optimized. During treatment, susceptible cells should filament considerably without showing cell lysis. A treatment that is too short results in many susceptible cells passing the filter, while a treatment that is too long results in much debris from lysed cells passing the filter and contaminating the sample.
11. When using a culture volume larger than 100 ml (*see* **Note 5**), the filter might get clogged and filtration might be too slow. To prevent this, divide the culture into smaller volumes and replace the filter in between filtration of these volumes. This is not necessary during the second filtration step, as most elongated cells should have been removed from the culture at this point.

Acknowledgments

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References

1. Shah D, Zhang Z, Khodursky AB et al (2006) Persisters: a distinct physiological state of *E. coli*. BMC Microbiol 6:53
2. Roostalu J, Jõers A, Luidalepp H et al (2008) Cell division in *Escherichia coli* cultures monitored at single cell resolution. BMC Microbiol 8:68
3. Sugimoto S, Arita-Morioka KI, Mizunoe Y et al (2015) Thioflavin T as a fluorescence probe for monitoring RNA metabolism at molecular and cellular levels. Nucleic Acids Res 43:e92
4. Keren I, Kaldalu N, Spoering A et al (2004) Persister cells and tolerance to antimicrobials. FEMS Microbiol Lett 230:13–18
5. Balaban NQ, Merrin J, Chait R et al (2004) Bacterial persistence as a phenotypic switch. Science 305:1622–1625
6. Keren I, Shah D, Spoering A et al (2004) Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. J Bacteriol 186:8172–8180

7. Johnson PJT, Levin BR (2013) Pharmacodynamics, population dynamics, and the evolution of persistence in *Staphylococcus aureus*. PLoS Genet 9:e1003123
8. Kwan BW, Valenta JA, Benedik MJ, Wood TK (2013) Arrested protein synthesis increases persister-like cell formation. Antimicrob Agents Chemother 57:1468–1473
9. Dörr T, Vulić M, Lewis K (2010) Ciprofloxacin causes persister formation by inducing the TisB toxin in *Escherichia coli*. PLoS Biol 8: e1000317
10. Windels EM, Ben Meriem Z, Zahir T et al (2019) Enrichment of persisters enabled by ß-lactam-induced filamentation reveals their single-cell awakening characteristics. Commun Biol 2:426

Chapter 5

Methods for Enrichment of Bacterial Persister Populations for Phenotypic Screens and Genomic Studies

Samantha Adikari, Elizabeth Hong-Geller, and Sofiya Micheva-Viteva

Abstract

Transient phenotypic adaptations in bacteria that enable survival at bactericidal antibiotic concentrations give rise to bacterial persistence. Naturally, the abundance of persister cells is very low (about 1 in 10^5 cells) in actively growing bacterial populations. Therefore, in order to study bacterial persistence mechanisms for therapeutics development, persister cells need to be enriched from a larger culture. Here, we describe three enrichment methods for obtaining *Burkholderia thailandensis* persisters: (1) flow sorting for persisters from exponentially dividing cultures by fluorescent staining of bacterial cells with a translational membrane depolarization-specific $DiBAC_4(3)$ dye, (2) antibiotic lysis of nonpersisters, and (3) culture aging to induce persister survival. We also describe herein the lysis of persister cells obtained by all three methods for downstream bacterial RNA extraction and transcriptomics analysis.

Keywords Bacterial persistence, Persister enrichment, Antibiotic tolerance, Stress response, Nutrient limitation, Culture aging

1 Introduction

Bacteria employ diverse mechanisms to overcome environmental stress conditions, including nutrient limitation and exposure to toxic metabolites. Phenotypic adaptations that increase bacterial survival under adverse conditions can also produce a small sub-population of transiently antibiotic tolerant cells. Current understanding of bacterial defense mechanisms causing antibiotic tolerance and persistence has been detailed in several review articles [1–3].

Various persister enrichment methods have been applied to study gene expression profiles responsible for the bacterial persistence phenotype. Bacteria exhibit high antibiotic tolerance in their stationary growth phase. Hence, aging of bacterial cultures is a simple means of naturally enriching persisters in a population

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[4, 5]. Other methods applied to enrich bacterial persisters include antibiotic lysis of susceptible bacteria [6, 7], and flow cytometry-based separation of genetically modified bacterial populations expressing dormancy markers [8]. It is notable that different stress conditions utilized to induce persistence in an experimental setting can unduly bias the outcome of phenotypic or gene activation assays. Thus, to obtain data representative of universal mechanisms that govern bacterial persistence, it is prudent to compare physiological and/or transcriptomic changes in response to a variety of different stressors that enrich the persister populations.

In a recently published comparative transcriptomics study [9], we demonstrated that *Burkholderia thailandensis* (*B.th.*) persister populations display gene expression profiles that are specific to the enrichment method used. Nevertheless, a small subset of functional and regulatory gene pathways are activated universally in persister populations independently enriched via two of three or all three methods. Finding core genes regulating the persister metabolic program will greatly improve the design of clinically relevant therapies to treat persistent bacterial infections.

Here we describe three distinct methods that we have previously applied to discover genes universally activated in *B.th.* persister populations: (1) flow sorting for persister cells exhibiting low proton motive force (PMF) in exponentially dividing populations; (2) lysing nonpersister cells with a beta-lactam antibiotic; and (3) inducing persister cell survival by exposing cultures to temperature stress and nutrient limitation. Although the culture and enrichment methods described herein have been applied to *Burkholderia* spp., which enter persistence at a higher rate than the majority of studied bacterial species [10–12], these methods could be utilized to enrich persister populations in other Gram-negative pathogens for various applications.

2 Materials

Prepare and store all materials and reagents at ambient room temperature unless otherwise specified. Refer to the MSDS of each chemical and commercial reagent for details on hazard characteristics, and follow waste disposal regulations accordingly. Note that sodium azide is not added to any preparations made in-house.

1. LB agar plates: Add 1 L of ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25 °C) and 40 g of LB agar powder to an autoclave-safe glass bottle, close the lid, and shake vigorously to dissolve the powder. Autoclave for 20 min at 121 °C and 18 psi. Transfer the bottle to a Class II biosafety cabinet (BSC), and pour hot agar into sterile petri dishes with lids (10 cm^2 rounds and 6 × 6

grid-marked squares), about 25 mL per plate. Allow agar plates to cool without lids for 1 h (*see* **Note 1**). Once agar plates have solidified and dried, replace the lids, and stack agar plates (lid-side-down) inside a plastic sleeve. Store at 4 °C for up to 1 month. Open only inside a Class II BSC.

2. LB Broth: Add 500 mL of ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25 °C) and 12.5 g of LB Broth powder to a glass bottle, close the lid, and shake vigorously to dissolve the powder. Transfer the bottle to a Class II BSC, and filter LB Broth through a vacuum-assisted 0.2μm PES membrane filter into a sterile receiver bottle. Store at ambient room temperature for up to 1 month. Open only inside a Class II BSC.
3. *Burkholderia thailandensis* E264 (BEI Resources) in 50% (v/v) glycerol stock, stored at −80 °C.
4. Liquid chromatography-mass spectrometry (LC-MS) grade dimethyl sulfoxide (DMSO).
5. 10 mg/mL working stock of meropenem: Dissolve 50 mg of meropenem powder in 5 mL of DMSO. Make single-use aliquots (50μL each) in sterile 200μL tubes, and freeze immediately. Store at −80 °C for up to 12 months (*see* **Note 2**).
6. 1 M/1 N NaOH (40 mg/mL).
7. 10 mg/mL working stock of ofloxacin: Dissolve 0.1 g of ofloxacin powder in 10 mL of 1 M NaOH. Make single-use aliquots (50μL each) in sterile 200μL tubes, and freeze immediately. Store at −80 °C for up to 12 months (*see* **Note 2**).
8. DNase/RNase-free distilled water.
9. 100μg/mL working stock of bis(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$): Dilute the 1 mg/mL commercial reagent in a 1:10 ratio in DNase/RNase-free distilled water. Store at −20 °C for up to 1 month.
10. 1× phosphate buffered saline (PBS) at pH 7.2, sterile, without Ca^{2+} or Mg^{2+} ions.
11. BacTiter-Glo Microbial Cell Viability Assay (Promega).
12. 5 mg/mL working stock of lysozyme: Dissolve 10 mg of lysozyme powder in 2 mL of 0.1 M KH_2PO_4. Make single-use aliquots (20μL each) in sterile 200μL tubes, and freeze immediately. Store at −20 °C for up to 12 months.
13. QIAzol Lysis Reagent (Qiagen) (*see* **Note 3**).
14. miRNeasy Mini Kit (Qiagen).
15. Chloroform.

3 Methods

Perform all procedures at ambient room temperature unless otherwise specified. For all procedures prior to bacterial cell lysis, perform all steps inside a Class II BSC to ensure the sterility of materials and samples. For two of the three enrichment protocols described herein (reduced PMF and antibiotic lysis of nonpersisters), we isolated persister cells from exponentially dividing *B.th.* cultures. These protocols could be similarly applied to isolate persisters from stationary-phase liquid cultures, although the minimal bactericidal antibiotic concentration and the duration of antibiotic exposure are both expected to increase, and therefore would need to be empirically determined for those cultures.

3.1 Preparation of Exponentially Dividing Bacterial Cultures

1. Prepare a new colony stock plate of *B.th.* E264. Using a sterile pipette tip, streak *B.th.* E264 from the glycerol stock tube onto an LB agar plate. Incubate the plate for 24 h at 37 °C. Store at ambient room temperature for up to 4 days (*see* **Note 4**).
2. Prepare a new stationary-phase liquid culture of *B.th.* E264. Using a sterile pipette tip, scrape a single colony of *B.th.* E264 from the colony stock plate, and resuspend into 5 mL of LB Broth in a 50 mL tube. Incubate the tube for 18 h at 37 °C with agitation at 220 rpm. Store at ambient room temperature for up to 8 h (*see* **Note 5**).
3. Prepare a new mid-log phase culture of *B.th.* E264. Dilute the stationary-phase liquid culture (made the previous day) in a 1:500 ratio into fresh LB Broth in a 50 mL tube or 250 mL conical culture flask, depending on the culture volume required. Incubate tube/flask for 4–5 h at 37 °C with agitation at 220 rpm. Use immediately for the relevant enrichment protocol when optical density at 600 nm (OD_{600}), as measured by a spectrophotometer, reaches 0.20–0.30 (*see* **Note 6**).

3.2 Determination of Minimal Bactericidal Antibiotic Concentration

Antibiotic treatments were performed using meropenem, a beta-lactam antibiotic that inhibits cell wall synthesis, or ofloxacin, a quinolone antibiotic that inhibits DNA replication. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of each new batch of antibiotics was determined prior to performing persister enrichment protocols. The MIC was calculated as the lowest antibiotic concentration at which the bacterial culture growth did not exceed the initial inoculation cell counts after 24 h of antibiotic exposure with aeration. The MBC was calculated as the antibiotic concentration at which a 50% loss of population viability was achieved.

1. Prepare 20 mL of a mid-log phase culture of *B.th.* E264 in a 50 mL Falcon tube (as described in Subheading 3.1, **steps 1–3**).
2. Thaw a single-use aliquot of antibiotic to test.
3. Label 5× 1.5 mL sterile tubes: 10, 7.5, 5, 2.5, and 1 mg/mL.
4. Prepare at least five different antibiotic stock concentrations: 10, 7.5, 5, 2.5, and 1 mg/mL. Use solvent that is optimal for each antibiotic type; for example, meropenem stock concentrations are made in DMSO, while ofloxacin stock concentrations are prepared in 1 M NaOH.
5. Mix the mid-log phase culture by inverting the 50 mL tube, then remove 1 mL of culture to measure the OD_{600}. When $OD_{600} = 0.20–0.30$, proceed to the next step.
6. Set aside 1 mL of mid-log phase culture to determine viable bacterial colony-forming units per mL culture (CFU/mL) corresponding to the bacterial density prior to antibiotic treatment (as described in Subheading 3.2, **steps 13–23**).
7. Label 6× 15 mL Falcon tubes: 10, 7.5, 5, 25, and 1μg/mL, untreated.
8. Mix the mid-log phase culture by inverting the 50 mL tube, then transfer 2 mL of culture to each labelled 15 mL tube.
9. Add 2μL of the corresponding antibiotic dilution to each tube (e.g., 2μL of 1 mg/mL stock into 1μg/mL tube).
10. Close the lids tightly and vortex the 15 mL tubes for 5 s to mix cultures thoroughly.
11. Incubate the 2 mL cultures for 24 h at 37 °C with agitation at 220 rpm.
12. Immediately perform cell survival counts (as described in Subheading 3.2, **steps 13–23**), first for the untreated mid-log phase culture (Subheading 3.2, **step 6**), and then for the treated cultures (Subheading 3.2, **step 11**) after 24 h of agitation.
13. Label a 6 × 6 grid-marked square LB agar plate with dilutions from 10^{-1} to 10^{-6}.
14. Transfer 200μL of bacterial culture from each treatment condition into the first row of a 96-well sterile U-bottom microtiter plate with lid.
15. Using a multichannel pipette, perform serial dilutions from 10^{-1} to 10^{-6} across the 96-well plate. Add 180μL of fresh LB Broth per well, then transfer 20μL of culture stepwise across the plate to achieve at ten-fold dilution in 200μL total, mixing thoroughly and changing pipette tips after each transfer.

16. Spot 10μL from each dilution (from 10^{-1} to 10^{-6}) for every sample onto the labelled square agar plate, changing the pipette tip after each spot.
17. Allow spots on the square agar plate to dry for 10 min undisturbed, with the plate lid on.
18. Wrap the 96-well plate (used for serial dilutions) tightly in Parafilm to minimize evaporation, and store at 4 °C until needed again for plating dilutions (for colony counting).
19. Incubate the square agar plate (lid-side-up) at 37 °C until bacterial colonies are clearly visible (24–36 h).
20. When colonies are visible on the square agar plate, determine the appropriate dilutions for colony counting (i.e., the dilutions showing about 5–20 distinct colonies per spot), and label a 10 cm^2 round LB agar plate for each treatment condition and dilution factor to be counted.
21. Retrieve the 96-well plate, transfer 100μL from each dilution to count onto the corresponding labelled round agar plate, and spread the culture evenly across the entire plate using an L-shaped sterile cell spreader.
22. Incubate the round agar plates (lid-side-down) at 37 °C until bacterial colonies can be easily counted (24–36 h) (*see* **Note 7**).
23. Calculate CFU/mL by multiplying the number of CFU by the dilution factor, corrected for the amount of bacterial culture plated on the round agar plate. For example, if 100μL from the 10^{-4} dilution factor was spread onto a round agar plate, and 56 CFU were enumerated on that plate, the cell count is 56×10^5 CFU/mL.
24. Determine the MBC as the lowest antibiotic concentration for which the percentage of cells surviving antibiotic treatment (CFU/mL) dropped below 50%, relative to the CFU/mL of the mid-log phase culture (from Subheading 3.2, **step 6**).

3.3 Enrichment Method #1: Flow Sorting for Persisters by Staining Membrane Depolarization

Bacterial cells maintain an electrical potential gradient, also known as a "proton-motive force" (PMF), across their cytoplasmic membrane in order to synthesize the essential high-energy metabolite adenosine triphosphate (ATP). Multidrug tolerance in bacterial persistence has been associated with depolarized bacterial cells, which are seen to exhibit below-average levels of PMF [13–15]. Fluorescence-activated cell sorting (FACS) can isolate persister cells from bacterial cultures treated with a PMF-sensitive dye. $DiBAC_4(3)$ is a bis-oxonal fluorescent dye that specifically permeates depolarized cells, binds to intracellular proteins or the cell membrane, and exhibits fluorescence with excitation and emission maxima at 490 and 516 nm, respectively.

While hyperpolarization of cells results in a rapid efflux of $DiBAC_4(3)$ and low fluorescence intensity (FI), cells with moderate-to-low PMF retain the dye and exhibit high FI. The final concentration of $DiBAC_4(3)$ used in the liquid culture media must be determined empirically to avoid dye-saturation. For Gram-negative bacteria, we found that a final concentration of 1μg/mL of $DiBAC_4(3)$ showed the best correlation between the percentage of antibiotic-surviving cells (persisters) and cells with high FI (low PMF). The high-$DiBAC_4(3)$ population can be sorted using the FACS technique described below. We found that a minimum of 10^6 sorted cells are required for lysis and subsequent bacterial RNA extraction. Collection of 10^6 sorted cells with high FI is a lengthy process (>4 h), given their low abundance (<1%) in exponentially dividing bacterial cultures. To minimize changes in the global transcriptome during this long sorting time, cells are best harvested and processed in batches staggered at time intervals not exceeding half the time of the average replication cycle for the particular bacterial species in use. As such, the following FACS technique is most efficiently performed as a two-person protocol.

1. Configure the FACS instrument with a blue laser and a FITC bandpass filter (excitation 488 nm, emission 530/30 nm). We used a FACSAria II flow cytometer (Becton Dickinson, NJ).
2. Prepare 25 mL of mid-log phase culture of *B.th.* E264 in a 50 mL Falcon tube (as described in Subheading 3.1, **steps 1–3**).
3. Mix the mid-log phase culture by inverting the 50 mL tube, then remove 1 mL of culture to measure the OD_{600}. when $OD_{600} = 0.20–0.30$, proceed to the next step.
4. Divide the remaining culture into 2× 50 mL tubes for stained (20 mL) and unstained (3 mL) conditions.
5. For the stained condition, add $DiBAC_4(3)$ to the culture in a 1:100 ratio to achieve a final concentration of 1μg/mL of $DiBAC_4(3)$ from a 100μg/mL working stock, and vortex for 5 s to mix.
6. Incubate the stained and unstained conditions for 30 min at 37 °C with agitation at 220 rpm.
7. Wash both conditions by adding 5 mL LB Broth per tube.
8. Pellet the cells by centrifugation at 5000 × *g* for 5 min, then discard the supernatant.
9. Repeat the wash and centrifugation steps with another 5 mL of LB Broth (as described in Subheading 3.3, **steps 7** and **8**).
10. Resuspend the cells from both conditions in 1 mL of LB Broth per tube to achieve a cell density (OD_{600}) of approximately

0.10, and proceed immediately with the cytometer setup and cell sorting (as described in Subheading 3.3, **steps 11** and **12**).

11. Setup the sorting parameters on the cytometer by performing hierarchical gating. Firstly, gate live cells on a cell distribution plot of the log-scale of side scatter-area (SSC-A) versus forward scatter (FSC). Secondly, gate stained cells on a cell distribution plot of the log-scale of fluorescence through the 530/30 emission filter versus FSC. Use the unstained cells as a negative control for $DiBAC_4(3)$ retention (*see* **Note 8**).
12. Sort persisters from nonpersisters until the full culture volume is exhausted, harvesting the persister cells in batches at 20 min intervals. For each batch of sorted persisters, proceed immediately with bacterial cell lysis (as described in Subheading 3.6).

3.4 Enrichment Method #2: Antibiotic Lysis of Nonpersister Cells

Beta-lactam antibiotics inhibit cell wall biosynthesis in bacteria and thus cause cell lysis in the course of one replication cycle. Even cell cultures originating from a single colony consist of subpopulations not synchronized in their growth rates. Antibiotic incubation for a period of time exceeding several replication cycles will achieve maximum enrichment for persisters that survive antibiotic-mediated lysis. While 24 h incubation in 2× MBC of beta-lactam antibiotic provides a highly enriched population of persisters, the quality of RNA isolated from cells exposed to antibiotics for a long period is not satisfactory for further downstream transcriptomics analyses. Therefore, to enrich persisters in exponentially dividing *B. th.* E264 cultures, we chose a shorter incubation time for 2× MBC of meropenem based upon measurements of metabolic index for the entire bacterial population. Though the measurement of metabolic index is optional, it can help to determine the shortest duration of antibiotic treatment necessary for complete lysis of nonpersister cells in the type of bacterial culture under investigation.

We determined metabolic index as a ratio of the total ATP pool in the bacterial population to the total cell number in the population. While cell number is derived from CFU/mL calculations (*see* Subheading 3.2, **step 23**), the total ATP pool can be determined with the BacTiter-Glo Microbial Cell Viability Assay (performed according to the manufacturer's instructions). The total ATP pool is measured in relative luminescence units (RLU) by spectrophotometer.

For *B.th.* E264, we determined the total ATP pool of mid-log phase cultures prior to meropenem exposure, then added meropenem treatment, and collected samples for ATP measurements at 30 min intervals over a 6 h incubation period. Upon exposure to 2× MBC of meropenem, the metabolic index (RLU/CFU) increased within the first 3 h, then rapidly dropped to 200% below the preexposure levels by 4 h. We also observed that these

cultures started to form precipitates and aggregates after 4 h treatment. The initial increase in metabolic index was due to an exponential increase of RLU coupled with no significant change in CFU/mL. By the 3 h measurement, RLU had dropped significantly while CFU/mL had decreased by only 50%. At 4 h posttreatment, the counts of viable bacteria had decreased by 80% compared to preexposure counts. It was therefore determined that 2× MBC of meropenem should be applied to *B.th.* E264 cultures for a period of 4 h to achieve efficient lysis of nonpersisters.

After the appropriate incubation period for antibiotic-induced cell lysis has been determined for the bacterial culture under investigation, proceed with persister isolation using the following enrichment protocol.

1. Prepare 25 mL of mid-log phase culture of *B.th.* E264 in a 50 mL Falcon tube (as described in Subheading 3.1, **steps 1–3**).
2. Mix the mid-log phase culture by inverting the 50 mL tube, then remove 1 mL of culture to measure the OD_{600}. When $OD_{600} = 0.20–0.30$, proceed to the next step.
3. Divide the remaining culture into 2× 50 mL tubes for treated (20 mL) and untreated (3 mL) conditions.
4. For the treated condition, add 2× MBC of meropenem and vortex for 5 s to mix.
5. For the untreated condition, add an equivalent volume of DMSO and vortex for 5 s to mix.
6. Incubate both tubes for 4 h at 37 °C with agitation at 220 rpm (*see* **Note 9**).

3.5 Enrichment Method #3: Culture Aging to Induce Persister Cell Survival

This method for persister population enrichment represents a long-term stationary phase model of population aging, which supports biofilm formation. Applying the protocol for persister flow sorting (as described in Subheading 3.3), we found that on average 60% of the *B.th.* E264 cells isolated from a 14-day-old stationary culture on an LB agar plate exhibited low PMF. For further enrichment of the persister population, we extended the culture aging period to 21 days. For samples collected from 21-day-old agar plates, the biofilm formation was such that it prevented the cell separation required for single-cell flow cytometry analysis of PMF. Beyond 21 days, bacterial colonies taken from aged agar plates and inoculated into fresh LB Broth did not consistently recover population cell growth. Additionally, the quality of RNA isolated from cultures older than 21 days was significantly deteriorated.

1. Prepare a new colony stock plate of *B.th.* E264 (as described in Subheading 3.1, **step 1**).

2. To age the culture, wrap the colony stock plate tightly in Parafilm and store for 21 days at 4 °C (*see* **Note 10**).
3. After 20 days of culture aging, prepare another new colony stock plate of *B.th.* E264 (as described in Subheading 3.1, **step 1**) to serve as a 1-day-old control; colonies from this fresh plate will be harvested and processed in parallel to the 21-day-old sample.
4. After 21 days of culture aging, harvest bacterial colonies from the 21-day-old and 1-day-old colony stock plates. Add 1 mL of PBS to 2× 2 mL tubes, scrape approximately 50–100 CFU from the agar surface with a sterile pipette tip, and resuspend the cells in PBS.
5. Proceed immediately with bacterial cell lysis (as described in Subheading 3.6).

3.6 Bacterial Cell Lysis with Lysozyme Pretreatment

Enzymatic degradation of the bacterial cell wall with lysozyme greatly improves the yield and quality of RNA extracted from persister cells.

1. Pellet the cells by centrifugation at 5000 × *g* for 5 min, then discard the culture supernatant.
2. Resuspend the cell pellet in 200μL of PBS.
3. Add 1000 U of lysozyme, vortex for 5 s to mix, then pulse spin.
4. Incubate the cells for 30 min in a 37 °C heat-block or water-bath, then pulse spin.
5. Add 700μL of QIAzol Lysis Reagent to lyse cells, and vortex for 5 s to mix.
6. Incubate the cell lysates for 15–30 min with rotation, then pulse spin.
7. Freeze the cell lysates at −20 °C for at least 12 h (*see* **Note 11**).
8. Proceed with bacterial RNA extraction (as described in Subheading 3.7).

3.7 Bacterial RNA Extraction

1. Thaw cell lysates (from Subheading 3.6, **step 7**) for 10–15 min on ice, then pulse spin.
2. Proceed with bacterial RNA extraction using reagents and protocol from the miRNeasy Mini kit. We refer to the *Qiagen miRNeasy Mini Handbook* for full details, available online at www.qiagen.com/HB-1277.

4 Notes

1. While the agar sets, keep the BSC running to maintain laminar air-flow; this will help remove condensation from the cooling agar, which would otherwise collect inside the plate lid.
2. Antibiotics stored for >12 months can begin to lose their potency.
3. QIAzol contains two dangerous components: phenol and guanidine thiocyanate. QIAzol is toxic by inhalation, in contact with skin, and if swallowed. Handle inside a chemical fume hood. Contact with acids liberates highly toxic gas. Do not combine with bleach. Suitable fire extinguishing agents include CO_2, extinguishing powder, water spray, and alcohol-resistant foam. Avoid inhaling gases liberated from explosion or combustion.
4. Colony stock plates that are used to make mid-log phase cultures must be discarded after 4 days to avoid genetic and/or phenotypic adaptation to low nutrient conditions. Plates should be wrapped tightly in Parafilm to minimize moisture loss. *B.th.* E264 colonies taken from plates >4 days old are sticky and difficult to resuspend into LB Broth.
5. Stationary phase liquid cultures (grown for 18 h) must be discarded after 8 h to avoid genetic and/or phenotypic adaptation to low nutrient conditions. Overnight cultures >8 h old can take longer to reach mid-log phase of growth when diluted in fresh LB Broth.
6. Freshly made mid-log phase cultures (OD_{600} = 0.20–0.30) will continue to grow at ambient room temperature, even without constant agitation, for about 30 min after the 37 °C incubation period. Cultures should therefore be removed from the shaking incubator about 30 min prior to treatment (as the OD_{600} approaches 0.20), then the OD_{600} should be measured again immediately prior to treatment. Do not allow mid-log phase cultures to overgrow, that is, $OD_{600} > 0.30$.
7. Do not allow colonies to grow very large, as neighboring colonies will begin to merge together and smaller colonies will be obscured; this makes it more difficult to obtain an accurate colony count.
8. Positive controls for $DiBAC_4(3)$ retention can also help to set the threshold gate for the collection of persisters with high FI levels. To make positive controls, use stationary-phase liquid cultures or bacteria treated with a metabolic inhibitor, then stain with $DiBAC_4(3)$ as described above.
9. Duration of antibiotic exposure must be empirically determined for each microbial species.

10. The low temperature allows for sufficient moisture retention within the agar medium to ensure cell viability throughout the aging period.
11. Freezing the cell lysates in QIAzol Lysis Reagent overnight improves the lysis efficiency and final RNA yield. For long-term storage, cell lysates should be stored at −80 °C to prevent RNA degradation prior to the bacterial RNA extraction step.

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References

1. Rocha-Granados MC, Zenick B, Englander HE et al (2020) The social network: Impact of host and microbial interactions on bacterial antibiotic tolerance and persistence. Cell Signal 75:109750
2. Van den Bergh B, Fauvart M, Michiels J (2017) Formation, physiology, ecology, evolution and clinical importance of bacterial persisters. FEMS Microbiol Rev 41:219–251
3. Gollan B, Grabe G, Michaux C et al (2019) Bacterial persisters and infection: past, present, and progressing. Annu Rev Microbiol 73:359–385
4. Leszczynska D, Matuszewska E, Kuczynska-Wisnik D et al (2013) The formation of persister cells in stationary-phase cultures of *Escherichia coli* is associated with the aggregation of endogenous proteins. PLoS One 8: e54737
5. Wood DN, Chaussee MA, Chaussee MS et al (2005) Persistence of *Streptococcus pyogenes* in stationary-phase cultures. J Bacteriol 187:3319–3328
6. Keren I, Minami S, Rubin E et al (2011) Characterization and transcriptome analysis of *Mycobacterium tuberculosis* persisters. mBio 2: e00100-00111
7. Keren I, Shah D, Spoering A et al (2004) Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. J Bacteriol 186:8172–8180
8. Shah D, Zhang Z, Khodursky A et al (2006) Persisters: a distinct physiological state of *E. coli*. BMC Microbiol 6:53
9. Micheva-Viteva SN, Shakya M, Adikari SH et al (2020) A gene cluster that encodes histone deacetylase inhibitors contributes to bacterial persistence and antibiotic tolerance in *Burkholderia thailandensis*. mSystems 5:e00609
10. Micheva-Viteva SN, Ross BN, Gao J et al (2018) Increased mortality in mice following immunoprophylaxis therapy with high dosage of nicotinamide in *Burkholderia* persistent infections. Infect Immun 87:e00592–e00518
11. Ross BN, Micheva-Viteva S, Hong-Geller E et al (2019) Evaluating the role of *Burkholderia pseudomallei* K96243 toxins BPSS0390, BPSS0395, and BPSS1584 in persistent infection. Cell Microbiol 21:e13096
12. Nierman WC, Yu Y, Losada L (2015) The *in vitro* antibiotic tolerant persister population in *Burkholderia pseudomallei* is altered by environmental factors. Front Microbiol 6:1338
13. Meylan S, Porter CBM, Yang JH et al (2017) Carbon sources tune antibiotic susceptibility in *Pseudomonas aeruginosa* via tricarboxylic acid cycle control. Cell Chem Biol 24:195–206
14. Harms A, Maisonneuve E, Gerdes K (2016) Mechanisms of bacterial persistence during stress and antibiotic exposure. Science 354: aaf4268
15. Liu Z, Hu K, Tang Y et al (2018) SmpB downregulates proton-motive force for the persister tolerance to aminoglycosides in *Aeromonas veronii*. Biochem Biophys Res Commun 507:407–413

Part III

Single-Cell Analysis of Persister Cells

Chapter 6

Observing Bacterial Persistence at Single-Cell Resolution

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Abstract

Within a bacterial population, there can be a subpopulation of cells with an antibiotic-tolerant persister phenotype characterized by long lag phase. Their long lag phase necessitates long (hours or days) periods of single-cell observation to capture high-quality quantitative information about persistence. We describe a method of single-cell imaging using glass bottom dishes and a nutrient agarose pad that allows for long-term single-cell microscopy observation in a stable environment. We apply this method to characterize the lag phase and persistence of individual *Escherichia coli* cells.

Keywords Persistence, Persisters, Single-cell microscopy, Lag time, Antibiotic resistance

1 Introduction

When a bacterial population is exposed to antibiotics, a subpopulation called persisters may survive while the majority of cells quickly die. Single-cell imaging has revealed that persister cells exhibit long lag times when transferred from stationary phase to fresh nutrient medium. That is, while most of the population rejuvenates and enters a reproductive state, this subpopulation remains in the non-dividing state for an extended period of time.

We designed a set of experiments to allow one to observe persistence at single-cell resolution and characterize the effect of stationary phase on lag times. The first experiment, determining the time delay in killing of growing cells (Subheading 3.1), captures the distribution of the time delay in killing growing bacteria in the presence of an antibiotic. Cells are exposed to ampicillin and the time of killing is determined using single-cell microscopy. The second experiment, single cell observation of lag time after starvation (Subheading 3.2), captures the distribution of lag times of a previously starved bacterial population. Cells are starved of nutrients (nongrowing state) and then transferred to a nutrient-rich

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environment. For each cell, the time at which it begins to grow (i.e., lag time) is determined using single-cell microscopy.

Both experiments require the observation of single cells over long periods of time (hours or days) within a stable environment. To achieve this, we make use of glass bottom microscopy dishes, which allow cells to be sandwiched in the bottom of the dish between a nutrient agarose pad and a cover glass. The nutrient agarose pad is thick and enclosed to maintain a consistent environment around the cells for the duration of the experiment, dramatically increasing the possible duration of single-cell microscopy experiments. Experiments using this method follow a basic protocol: a nutrient agarose pad is prepared for the experiment, a liquid culture is sandwiched in a glass bottom dish beneath the nutrient agarose pad, the dish is mounted on a phase-contrast microscope, single-cell images are captured for ~20 h and the resulting images are analyzed using the MicrobeJ plugin for ImageJ. The specific protocols for each experiment are outlined in Subheadings 3.1 and 3.2, with in-depth descriptions of all the shared procedures provided in Subheadings 3.3–3.6.

2 Materials

Prepare all solutions using nanopure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ·cm) and analytical grade reagents. Prepare and store all reagents at room temperature, unless otherwise indicated.

2.1 Determining Time Delay in Killing of Growing Cells

1. *E. coli* K12 NCM3772 Δ*motA* strain, stored in 25% (v/v) glucose solution at −80 °C.
2. Luria–Bertani (LB) broth: Add 600 mL water to a 1 L glass medium bottle with screw cap. Weigh 15 g of Luria–Bertani broth powder and transfer to the bottle. Close cap tightly and mix by inverting and swirling the bottle until the powder is completely dissolved. Loosen cap and autoclave at 121 °C for 30 min. Allow the solution to cool before tightening cap.
3. 100 mg/mL ampicillin solution in water, stored at 4 °C for up to a week.
4. 1 mM propidium iodide (PI) solution in water, prepare fresh.
5. Agarose powder.
6. 10 cm petri dish.
7. Glass culture tubes capable of holding 5 mL of culture and compatible caps (typical size is 20 mm).
8. 20 mL glass, screw-top scintillation vial.
9. Sterile P1000 pipette tips and compatible pipette.

10. Sterile 15 mL conical tube.
11. 35 mm glass bottom dish with a 20 mm well (*see* **Note 1**).
12. Metal scoopula (*see* **Note 2**).
13. Parafilm M laboratory film.
14. Optional: Cling film if storing pad overnight.
15. Spectrophotometer with standard cuvette.
16. Water bath, shaking at 250 rpm, 37 °C.
17. Inverted microscope fitted with an automated mechanical XY stage, 60× objective, TRITC filter, and autofocus, housed in an incubator at 37 °C.
18. ImageJ image analysis software with MicrobeJ plug-in (*see* **Note 3**).

2.2 Single-Cell Observation of Lag Time After Starvation

1. *E. coli* K12 NCM3772 Δ*motA* strain, stored in 25% (v/v) glucose solution at −80 °C.
2. N^-C^- minimal medium: Dissolve 1 g K_2SO_4, 13.5 g K_2HPO_4, 4.7 g KH_2PO_4, 0.048 g $MgSO_4$, and 2.5 g NaCl in 1 L water and filter through a 0.22 μm pore MilliporeSigma filter. Store at 4 ° C (*see* **Note 4**).
3. Starvation medium: Mix 1 L N^-C^- minimal medium with 2.1 g ammonium chloride. Adjust pH to 7.
4. Glucose minimal medium: Mix 960 mL N^-C^- minimal medium with 2.1 g ammonium chloride and 40 mL of 1 M glucose solution in water. Adjust pH to 7.
5. Luria–Bertani (LB) broth: Add 600 mL water to a 1 L glass medium bottle with screw cap. Weigh 15 g of Luria–Bertani broth powder and transfer to the bottle. Close cap tightly and mix by inverting and swirling the bottle until the powder is completely dissolved. Loosen cap and autoclave at 121 °C for 30 min. Allow the solution to cool before tightening cap.
6. 100 mg/mL ampicillin solution in water, stored at 4 °C for up to a week.
7. Agarose powder.
8. 10 cm petri dish.
9. Glass culture tubes capable of holding 5 mL of culture and compatible caps (typical size is 20 mm).
10. 20 mL glass, screw-top scintillation vial.
11. Sterile P1000 pipette tips and compatible pipette.
12. Sterile 15 mL conical tube.
13. 35 mm glass bottom dish with a 20 mm well (*see* **Note 1**).
14. Metal scoopula (*see* **Note 2**).

15. Parafilm M laboratory film.
16. Optional: Cling film if storing pad overnight.
17. Spectrophotometer with standard cuvette.
18. Water bath, shaking at 250 rpm, 37 °C.
19. Inverted microscope fitted with an automated mechanical XY stage, 60× objective, and autofocus, housed in an incubator at 37 °C.
20. ImageJ image analysis software, with MicrobeJ plug-in (*see* **Note 3**).

3 Methods

3.1 Determining Time Delay in Killing of Growing Cells

1. Prepare a seed culture by taking cells from a −80 °C frozen stock and culturing in fresh LB overnight.
2. The following morning, transfer cells from the seed culture to fresh LB and culture for at least nine doublings, to an OD_{600} of 0.2–0.3 (experimental culture).
3. Following the protocol described in Subheadings 3.3 and 3.4, prepare an agarose pad of LB + 1% agarose + 100 μg/mL ampicillin + 4 μM PI (*see* **Note 5**) and use it to sandwich 4 μL of the experimental culture in a glass bottom dish. Seal the glass bottom dish with Parafilm and mount on a microscope with incubator set to 37 °C.
4. Images of the cells should be captured in a time lapse using phase contrast and a TRITC filter in 10-min time intervals, and a duration of ~20 h. *See* Subheading 3.5.
5. Time-lapse images can then be analyzed in MicrobeJ, as described in Subheading 3.6, to determine the time of death for each cell. We found PI to be a good indicator of death by ampicillin (*see* **Note 6**), however, because it stains nucleic acids, it does not identify lysed cells. Thus, we measured the time at which each cell either was stained by PI or lost its phase-contrast refractivity as the time of death (since lysed cells lose their refractivity due to the loss of intracellular materials). Example images can be found in [1].

3.2 Single-Cell Observation of Lag Time After Starvation

1. Take cells from a −80 °C frozen stock and culture in LB for 4–6 h (seed culture).
2. Transfer cells from the seed culture to glucose minimal medium at very low density (OD_{600} of ~0.0001 or below) and culture overnight (preculture) (*see* **Note 7**).
3. Dilute the preculture 20–50× into fresh glucose minimal medium and allow cells to grow exponentially for at least four doublings (experimental culture).

4. Wash the experimental culture. Begin by adding 5 mL of experimental culture to a prewarmed sterile 15 mL conical tube.
5. In a centrifuge prewarmed to 37 °C, centrifuge the experimental culture at 1900 rcf for 5 min.
6. Discard supernatant and resuspend cells in 5 mL of starvation medium.
7. Repeat **steps 5** and **6**.
8. Transfer cells to a sterile glass culture tube and culture in starvation medium (starvation culture).
9. Following the protocol described in Subheadings 3.3 and 3.4, prepare an agarose pad with glucose minimal medium + 1% agarose + 100 μg/mL ampicillin and use it to sandwich 5 μL of the starvation culture in a glass bottom dish. Seal the glass bottom dish with Parafilm and mount the dish on a microscope with incubator set to 37 °C.
10. Images of the cells should be captured in a time-lapse using phase-contrast exposure, 10-min time intervals, and a duration of ~20 h, *see* Subheading 3.5.
11. Time-lapse images can then be analyzed in MicrobeJ, as described in Subheading 3.6, to measure cell length in each frame. The lag time of each cell is then determined by the time after sandwiching at which its length begins to increase (*see* **Note 8**).

3.3 Preparing the Agarose Pad

1. Prewarm a rack of P100 pipette tips (*see* **Note 9**).
2. Add 3 mL of medium and 0.3 g agarose to a 20 mL glass, screw-top scintillation vial. Screw the cap loosely closed (to allow venting) and place the vial in a microwave. Warm the medium in short (<1 s) pulses, bringing the medium just to boiling in each pulse before stopping the microwave. Every few pulses, remove the vial and swirl gently (*see* **Note 10**), making sure that the medium does not touch the cap. Continue until all agarose crystals have dissolved.
3. Allow the medium to cool for ~1 min to 55–65 °C (warm, but not painful to the touch). Gently turn the vial to collect any condensation back into the medium, again being careful that the medium does not touch the cap.
4. Add 100 μg/mL ampicillin and use a pipette with prewarmed tip to mix by pipetting up and down gently, being careful not to introduce bubbles. For determining the time delay in killing of living cells (Subheading 3.1), also add 4 μM PI.
5. Using a pipette with prewarmed pipette tips, transfer 2.5 mL of medium to a glass bottom dish, being careful not to introduce any bubbles. Return the lid to the dish.

6. Allow the pad to cool for 4 h at room temperature.
7. The pad may be used immediately or stored for up to 24 h. For storing, wrap the edge of the dish in Parafilm and cover with cling film, store at 20 °C.

3.4 Sandwiching Cells

1. Unwrap the glass bottom dish (if stored overnight). Sterilize a scoopula with 100% ethanol and dry.
2. Gently remove the agarose pad from the glass bottom dish using the sterile scoopula. Run the scoopula along the wall of the dish to release the pad and then lift the pad out of the dish on the scoopula. Return the pad to the glass bottom dish.
3. Place the glass bottom dish into the base of a 10 cm Petri dish, crack the lid of the glass bottom dish, and place in an incubator at 37 °C to prewarm for 30 min (*see* **Note 11**). Prewarm the microscope enclosure to 37 °C.
4. Use a sterile scoopula to gently lift the prewarmed agarose pad out of the glass bottom dish, and balance as securely as possible on the scoopula. Using two pieces of tape, secure the scoopula to a test-tube rack (or similar) so that the end with the agarose pad is suspended in the air, being careful to prevent the agarose pad from touching any surfaces.
5. Dry the inside of the glass bottom dish using microfiltered forced air, if available.
6. Pipette 5 μL of liquid culture into the well of the glass bottom dish, using several droplets to spread the cells across the entire well, but not outside the well.
7. Remove tape to free the scoopula and place the agarose pad back into the glass bottom dish, running the scoopula along the edge to lower it as evenly as possible. Use the scoopula to gently press the pad down to remove any bubbles (*see* **Note 2**).
8. Wrap the edge of the dish in Parafilm (*see* **Note 12**).

3.5 Microscopy

1. Mount the glass bottom dish onto the microscope with an enclosure prewarmed to 37 °C. We use an oil-immersed 60× objective. The objective should be high enough magnification to facilitate accurate segmentation of individual cells.
2. To prepare the time-lapse, scan across the well of the dish to select positions at random for imaging. Positions should be centered around a cell, be nonoverlapping, and be spread as evenly as possible throughout the well. The time-lapse should be set to capture images of each position twice per doubling (~10 min for this experiment) with phase-contrast imaging. For determining the time of killing in living cells (Subheading 3.1), a red florescence exposure should also be used with a TRITC filter.
3. The time lapse should be set to run for 20 h (*see* **Note 13**).

3.6 MicrobeJ Analysis

1. Load a phase-contrast image, along with any corresponding exposures, into ImageJ and open MicrobeJ by selecting "Plugins"→"MicrobeJ"→"MicrobeJ."
2. Under the "Bacteria" tab, select the phase-contrast image to segment the bacteria. Checking the "shape" and "intensity" options will provide information about the shape (length, area, aspect ratio, etc.) and intensity (max, min, average intensity within each cell for each channel) (*see* **Note 14**).
3. Use the pencil icon in the bottom left of the "bacteria" tab to detect the cells and open the manual editing interface. This will overlay the phase-contrast image with the outlines of cells it has detected. In the manual editing interface, you can check and correct the segmentation by outlining any cells that have been missed, removing incorrectly detected cells, and adjusting the segmentation of cells (splitting two cells that have been outlined as one, adjusting the boundary of cells that have been improperly outlined, etc.) (*see* **Note 15**).
4. Once cells have been satisfactorily segmented, view the results using the MicrobeJ results table (bar graph icon in the top bar of the experiment editor). Here you will be able to view and save information about the position, shape, intensity, and so on of the cells you have segmented.

4 Notes

1. We use CellVis 35 mm glass bottom dishes. You will need the appropriate cover glass thickness for your microscope.
2. Bending one end of the scoopula so it has a foot of ~1 cm perpendicular to the handle helps to provide a gentle way to adjust (especially, to press down) the agarose pad.
3. ImageJ can be downloaded for free at: imagej.nih.gov/ij. The MicrobeJ plug-in can be downloaded for free at: microbej.com.
4. This solution may be made at 4× concentration and stored at −20 °C for convenience.
5. This concentration was chosen because it has minimal impact on cell viability in starvation while still being detectable.
6. We confirmed that PI is a good indicator for cell death by ampicillin by incubating cells with ampicillin and PI for 80 min before spreading them on an LB agar plate (no ampicillin). Across five biological replicates a total of ~25 PI+ cells were examined, and none grew. We additionally used a fluorescent glucose analog (2-NBDG) alongside PI to compare metabolic activity (uptake of 2-NBDG) and PI staining in

cells exposed to ampicillin. We found two distinct populations of cells, those that were PI stained with no uptake of 2-NBDG and those that were not PI stained with uptake of 2-NBDG, further confirming PI as a good indicator of cell death by ampicillin. More information can be found in [1].

7. The culture should still be growing exponentially the next morning.
8. An example of the results is given in [2].
9. The temperature of prewarm is not especially important for this step. Having the pipette tips warm simply helps to keep the agarose warm while transferring the agarose to the glass bottom dish and prevents the agarose from solidifying on the walls of the pipette tip. We prewarm to 80 °C, but even a prewarm to 37 °C would be better than using room temperature tips and may be more readily available.
10. An 11 × 21 cm Kimwipe (or similar), folded twice longwise ("hotdog" style) makes a good sling to wrap around the vial, so you do not need to hold the hot vial.
11. The agarose pad should be prewarmed long enough to come to 37 °C and ideally no longer. In our lab conditions, 30 min is optimal, but you may need to adjust the prewarming time. Use a consistent prewarming time to prevent differences in the pad due to different levels of drying.
12. Parafilm is gas-permeable with low water permeability. It allows oxygen and carbon dioxide to pass while preventing drying up the agarose pad.
13. For these experiments, 20 h was sufficient, but an experiment may be run for 48 h or longer. This is a major benefit of using the agarose pad to sandwich cells in the glass bottom dish.
14. We find that the "Local Default" segmentation method works well. If MicrobeJ is having difficulty segmenting your cells correctly, you will want to try using different segmentation methods. Some segmentation methods are computationally heavy, so it is helpful to use the "Rectangle" tool of ImageJ to select a smaller section of the image for MicrobeJ to work on while you are testing methods.
15. If MicrobeJ is consistently accepting noncells as cells or vice versa, you can adjust the segmentation parameters in the "Bacteria" tab. This allows you to set minimum and/or maximum values for the shape and intensity of objects it accepts as cells (area, length, intensity, etc.).

References

1. Şimşek E, Kim M (2018) The emergence of metabolic heterogeneity and diverse growth responses in isogenic bacterial cells. ISME J 12:1199–1209
2. Şimşek E, Kim M (2019) Power-law tail in lag time distribution underlies bacterial persistence. Proc Natl Acad Sci U S A 116: 17635–17640

Chapter 7

Phenotypic Characterization of Antibiotic Persisters at the Single-Cell Level: From Data Acquisition to Data Analysis

Nathan Fraikin, Laurence Van Melderen, and Frédéric Goormaghtigh

Abstract

Persister cells are present at low frequency in isogenic populations. Moreover, they are only distinguishable from the bulk at the recovery time, after the antibiotic treatment. Therefore, time-lapse microscopy is the gold-standard method to investigate this phenomenon. Here, we describe an exhaustive procedure for acquiring single-cell data which is particularly suitable for persister cell analysis but could be applied to any other fields of research involving single-cell time-lapse microscopy. In addition, we discuss the challenges and critical aspects of the procedure with respect to the generation of robust data.

Keywords Persistence, Microfluidics, Microscopy, Single-cell analysis

1 Introduction

Bacterial persisters are rare individuals within clonal populations capable of surviving lethal doses of bactericidal antibiotics. The scarce, unstable and variable nature of these phenotypic variants represents a barrier in the understanding of persister cells physiology [1]. Persister cells cannot be isolated from the bulk of the population due to the current absence of specific markers. Consequently, persister-distinctive traits are hidden among a vast majority of nonpersister cells and cell-to-cell heterogeneity is averaged out. While enrichment of persister cells can amplify these signals, they fail to capture persister cells in their native physiological state as they often rely on genetic mutations [2] or cell-sorting of cells in a specific metabolic state [3, 4]. Transition from population to single-cell analysis is thus necessary to interrogate persister specificities and understand the molecular mechanisms underlying the so-called persister switch. More specifically, time-lapse microscopy associated with fluorescent biosensors makes it possible to investigate bacterial processes non-invasively at the single-cell level over time.

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Recent advances in the expanding field of microfluidics have provided scientists with a number of tools for long-term live-imaging of bacterial populations at the single-cell level [5]. The Mother machine [6] and the CellASIC system (commercially available from Merck Millipore) are among the most popular microfluidic devices so far, allowing to record thousands of cells for tens to hundreds of generations within a controlled environment. Post-processing further allows segmentation and tracking of single-cells, thereby providing access to crucial parameters for persister cell analysis such as microcolony and single-cell growth rates, reconstruction of cell lineages, and tracking of intracellular fluorescence. Numerous complex software packages have been developed for this purpose during the last decade and are freely available, alleviating most of the difficulties with regards to the analysis of large image datasets.

Single-cell microscopy is a powerful and unique tool for studying different subpopulations of cells defined by the parameters described in the previous section. Flow cytometry-like analyses can be performed on cells with the main advantage that scientists can visualize the cells of interest and track down their fate through the entire span of the experiment: growth, antibiotic treatment and recovery. Comparison of different subpopulation of cells was for instance done in [7] to show that persisters to ofloxacin were not different in terms of growth rate prior antibiotic treatment as compared to non-persister cells. However, one could imagine much more complex and informative analyses using multiple fluorescent reporters in live cells and generating correlations between different physiological processes and bacterial growth rates in persister cells as well as in non-persister cells. Another unique benefit of single-cell microscopy is the potential to observe stochastic switching in live cells, a phenomenon initially described as central for bacterial persistence [2], but never directly visualized in vivo, despite significant efforts made in trying to link stochastic switching of toxin-antitoxin (TA) system activation and ppGpp synthesis with bacterial persistence to antibiotics as reviewed in [8].

2 Materials

1. MOPS medium [9]: 40 mM morpholinopropane sulfonic acid (MOPS), 4 mM Tricine, 10μM ferrous sulfate, 9.52 mM ammonium chloride, 276μM potassium sulfate, 0.5μM calcium chloride, 526μM magnesium chloride, 50 mM sodium chloride, 3 nM ammonium heptamolybdate, 0.4μM boric acid, 30 nM cobalt chloride, 10 nM cupric sulfate, 80 nM manganese chloride, and 10 nM zinc sulfate. Prepare a sterile filtered 10× stock, adjust pH to 7.4 with potassium hydroxide. Make 1× medium by mixing 10× stock, water, 1.32 mM

dipotassium phosphate and 0.4% glucose. Other less defined media (e.g., LB, M9) can be used as well, although it has been showed that persistence frequency is highly variable from one experiment to the other, particularly in rich non-defined LB medium [10].

2. 125 mL Erlenmeyer flasks with Kapsenberg aluminum caps.
3. Lysogeny agar (LA) plates: 5 g/L yeast extract, 10 g/L tryptone, 5 g/L sodium chloride, 15 g/L agar, autoclave for 30 min at 121 °C. Allow the medium to cool to 55 °C and pour plates (20–30 mL for 90 mm diameter plates).
4. Antibiotic stock solutions: 100 mg/mL ampicillin, 5 mg/mL ofloxacin. Add concentrated acid to dissolve ofloxacin. Keep antibiotics at 4 °C for use and store at −20 °C.
5. Inverted microscope with a heating solution at appropriate temperature (e.g., 37 °C), a microplate-compatible motorized stand and a hardware autofocus solution (e.g., Zeiss Definite Focus, Nikon Perfect Focus).
6. A commercial microfluidic perfusion system (e.g., CellASIC ONIX, Merck, with B04a plates).
7. Phosphate-buffered saline (PBS): 136.9 mM sodium chloride, 2.7 mM potassium chloride, 8.9 mM disodium phosphate, 1.5 mM monopotassium phosphate.
8. Bacterial strain: *Escherichia coli* MG1655.

3 Methods

3.1 Data Acquisition

Data acquisition represents the first part of the pipeline presented in Fig. 1, comprising inoculation of an exponentially growing culture, followed by inoculation of the microfluidic device and acquisition of time-lapse fluorescent microscopy images. As an example, the following section details the protocol for starting such experiment in the commercially available CellASIC microfluidic device.

3.1.1 Day 1: Preparation of Cultures and Medium

1. Prepare MOPS medium.
2. On a fresh LA plate, streak a frozen stock of the strain of interest with a sterile loop or toothpick. Incubate overnight at 37 °C.

3.1.2 Day 2: Precultures

1. Transfer 10 mL of MOPS medium to 125 mL Erlenmeyer flasks.
2. Pick a fresh colony from the plate and suspend it in the flask containing MOPS medium.
3. Incubate overnight at 37 °C with shaking (180–250 rpm, ~0.4 rcf).

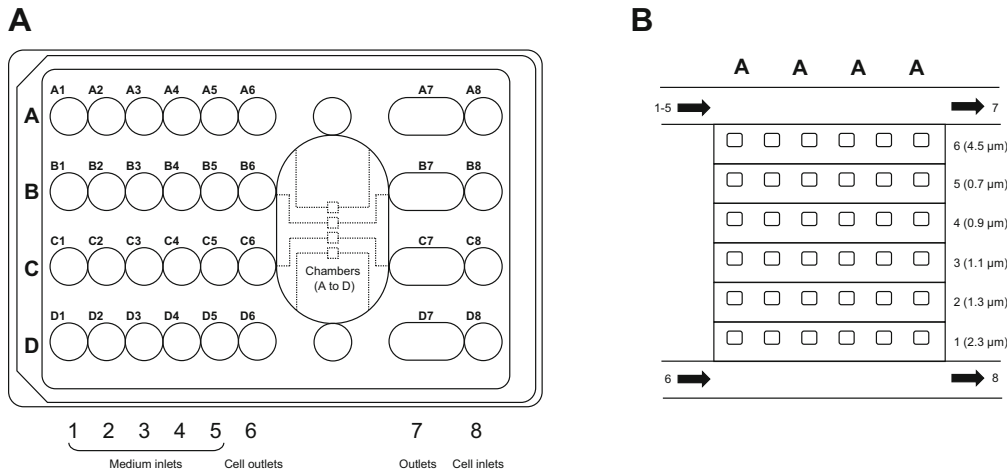

Fig. 1 Schematic representation of a B04a plate. (**a**) General view of the plate. Well columns 1–5 are medium inlets that are used to perfuse the cells during the experiment. Well columns 8 and 6 are cell inlets and outlets, respectively, and are used to inoculate the chambers. Well column 7 consists of outlets where medium from the perfusion will flow to. Each line of inlets and outlets (A, B, C, and D) perfuses one chamber for a total of four chambers (located by dashed squares). (**b**) Schematic representation of a chamber in the *xz* plane. The letter corresponding to the inlet/outlet line is printed above each chamber. The cell flow (inlet 8, outlet 6) is located at the bottom of the chamber while the medium flow (inlets 1–5, outlet 7) is located above the chamber. Each of these chambers is subdivided in six subchambers, each with a different ceiling height

3.1.3 Day 3: Cultures and Microscopy

1. For each culture to be diluted, prewarm a 125 mL flask containing 10 mL of MOPS medium and a screw-cap tube containing 1 mL of MOPS medium at 37 °C.
2. Measure the OD_{600} of the overnight cultures and dilute them to OD_{600} 0.03.
3. While the cultures are growing, prewarm the microscope at the desired temperature (e.g., 37 °C) (*see* **Note 1**).
4. In sterile tubes, aliquot 2 mL of MOPS medium and add 2µL of the desired antibiotic 1000× stock (e.g., ofloxacin to a concentration of 5µg/mL).
5. Drain all the storage solution from each well of the microfluidics plate using a pipette (*see* **Note 2**).
6. Add 300µL of MOPS medium to medium inlets (well columns 1–5, Fig. 1a).
7. Add 100µL of PBS to cell inlet (well column 8, Fig. 1a).
8. Seal the CellASIC manifold to the plate using the seal button from the CellASIC pressure controller, gently pressing the lid against the plate. A LED marked with "Seal" on the pressure controller will turn on to report a successful seal.
9. In the Manual Mode tab of the CellASIC Software, run the Liquid Priming Sequence.

10. After the Liquid Priming Sequence, press the seal button and remove the manifold.
11. Empty medium inlets 2 and add 300μL of MOPS medium with appropriate antibiotics (Fig. 1a).
12. Lid the plate and incubate statically at 37 °C. Prewarm 1 mL of PBS at 37 °C as well.
13. In the Protocol Editor tab of the CellASIC software, construct a program based on the following:
    (a) Open well group 1, Set pressure to 10 kPa for 5 h (Growth phase).
    (b) Open well group 2 for 5 h (Antibiotic phase).
    (c) Open well group 3, 4, and 5 for 30 h (Regrowth phase).
14. When cultures reach an OD_{600} of 0.5 (4 h 30 min to 5 h after inoculation), add 20μL of cells to 1 mL of prewarmed MOPS medium. Add 100μL of this cell suspension to cell inlets.
15. Seal the manifold to the plate as described above (**step 8**).
16. Load the plate on the microscope using an appropriate phase contrast objective and bring to focus at the position of the chambers (*see* Fig. 1a for the position of the chambers).
17. In the Manual Mode tab of the CellASIC software, start the Cell Loading Sequence. At the end of the sequence, *E. coli* cells should be visible in the chamber (*see* **Note 3**).
18. In the Manual Mode of the CellASIC software, press the Custom Sequence button and start the following sequence to wash out untrapped cells.
    (a) Open well group 1, Set pressure to 40 kPa.
19. Select as many microscopic fields as possible to image between 0.05 to 0.5 mm^2 of field surface (typically 5–50 fields of view). Set up a time-lapse, taking a phase contrast picture (plus any required fluorescence images) 3–4 times per generation, which equates to every 10–20 min with *E. coli* growing in MOPS medium with glucose as a carbon source (*see* **Note 4**).
20. In the Run tab of the CellASIC software, press the Start Run button. The rest of the image acquisition should now be fully automated (*see* **Note 5**).

3.2 Data Analysis

Time-lapse microscopy generates large multidimensional images, easily reaching hundreds of gigabytes, the processing of which constitutes a major bottleneck in the entire analysis pipeline. Figure 2 illustrates the classical steps required to process time-lapse microscopy data. First, cells are segmented and tracked, then morphology and fluorescence signals are quantified on the entire span of the experiment using complex algorithms. Next, key parameters for persistence are extracted and plotted. The next sections describe

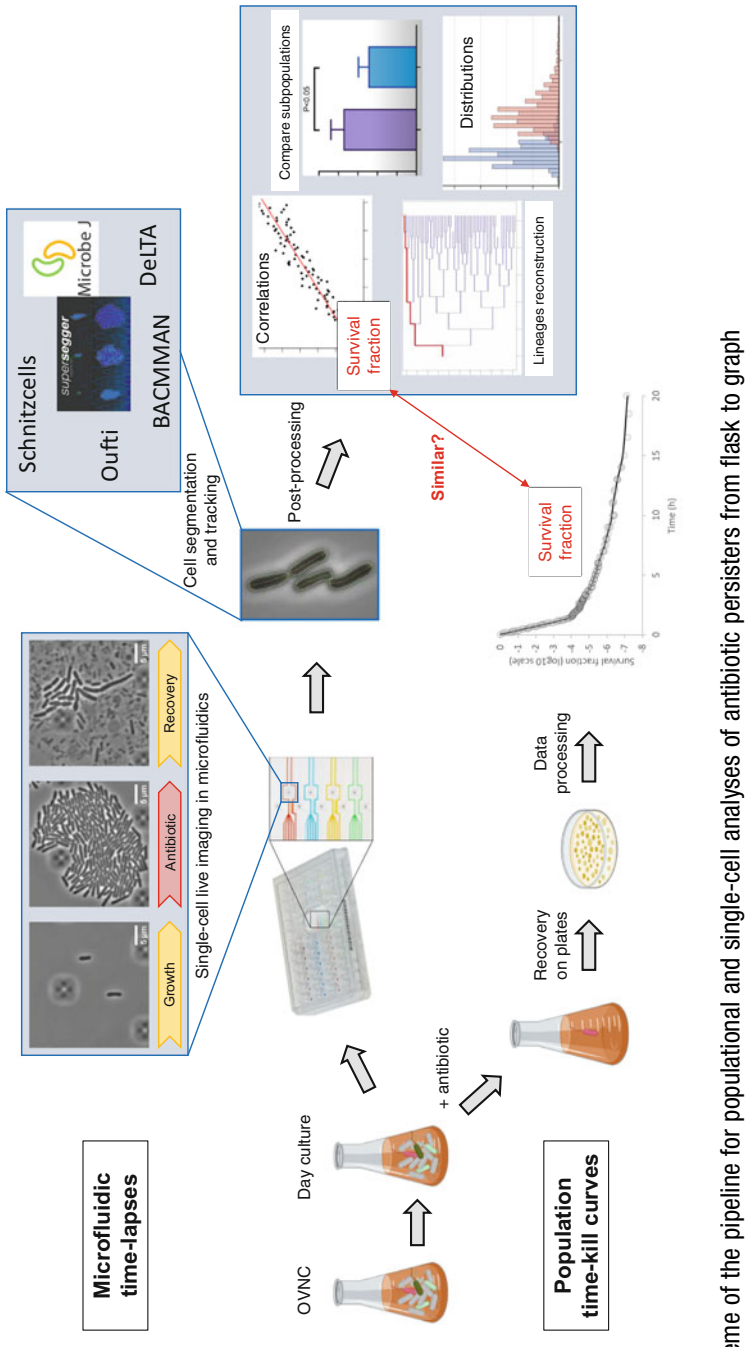

Fig. 2 Scheme of the pipeline for populational and single-cell analyses of antibiotic persisters from flask to graph

these steps and provide advices/suggestions regarding persister cells analysis.

3.2.1 Cell Segmentation, Cell Tracking and Intensity/Morphology Measurements

Within the past decade, a large set of specialized software packages have been developed to alleviate the major bottleneck associated with image data processing. A nonexhaustive list of software packages, with a brief description and their references is displayed in Table 1. All software packages offer reliable semiautomated analysis of large 2D time-series image stacks, including segmentation and tracking of cells, lineage reconstruction and cell characterization in terms of morphology, fluorescence and cell genealogy. They are freely available under the condition that the original paper is cited in the publication. An additional toolbox for postprocessing was developed by the Veening lab in the form of an R-package allowing advanced analysis and visualization of data collected through cell segmentation softwares [18]. Altogether, researchers have now access to a diversified toolbox for processing and postprocessing of large sets of images, significantly facilitating analysis of the physiology and heterogeneity at the single-cell level, an invaluable tool for the study of persister cells.

3.2.2 Defining and Monitoring Key Parameters for Persistence

Bacterial persistence is intimately linked with the growth stage and rate as extensively shown previously [1–3]. Cell-to-cell heterogeneity in growth rate and stage is therefore key to understand persistence to antibiotics. Monitoring both parameters requires tracking of cells during the entire experimental cycle which is composed of (1) growth before addition of the antibiotic, (2) antibiotic exposure, and (3) cell recovery after antibiotic removal (*see* **Note 6**).

3.2.3 Persister Identification

Persister cells are easily identified by their ability to recover after the antibiotic treatment. Any cell achieving multiple division cycles successfully during the recovery period is by definition a persister cell. Due to the low persister frequency (1–0.001% depending on the experimental setup), very large images (0.05–0.5 mm^2) might be needed to identify a significant number of such cells. However, as described in [7], microfluidic devices allow for high-throughput live-cell imaging, making it possible to identify significant numbers of persisters to apply statistical analyses even in setups in which the persister frequency is very low.

3.2.4 Microcolony Growth Rate

The cumulative surface of all cells within a microcolony is monitored through time, yielding a microcolony growth curve as illustrated in Fig. 3. The growth rate is defined as the slope during exponential growth phase, similarly as in flask cultures. Units are h^{-1} and this metric is easily converted into generation time (in hours) through the following formula: $t_{gen} = \frac{\ln(2)}{\text{growth rate}}$. This metric can be used to compare different microcolonies between each other and should also be used to verify that the growth rate of

Table 1
Nonexhaustive comparison of the most popular single-bacterial cell image analysis software packages

Software	Language	Specificities	References
MicrobeJ	ImageJ	Plugin with an interactive and user-friendly interface that benefits from the extensive library of functions and plugins available in ImageJ	[11]
SuperSegger	Matlab	General purpose machine-learning based tool, very reliable for segmentation of large microcolonies	[12]
DeLTA	Python	Deep learning-based analysis designed for *E. coli* grown in a mother machine device	[13]
Oufti MicrobeTracker	Standalone Matlab	General-purpose cell tracking software for cell and intracellular spots detection and tracking that comes with a graphical interface allowing users to interact with segmentation processes	[14, 15]
BACMMAN	Java	Specifically designed for the mother machine	[16]
Schnitzcells	Matlab	Specifically designed to analyze fluorescent time-lapse image of *E. coli* grown on agarose pads	[17]
BactMAP	R	R package for analysis and visualization of cell segmentation and cell fluorescence data acquired through independent segmentation software packages	[18]

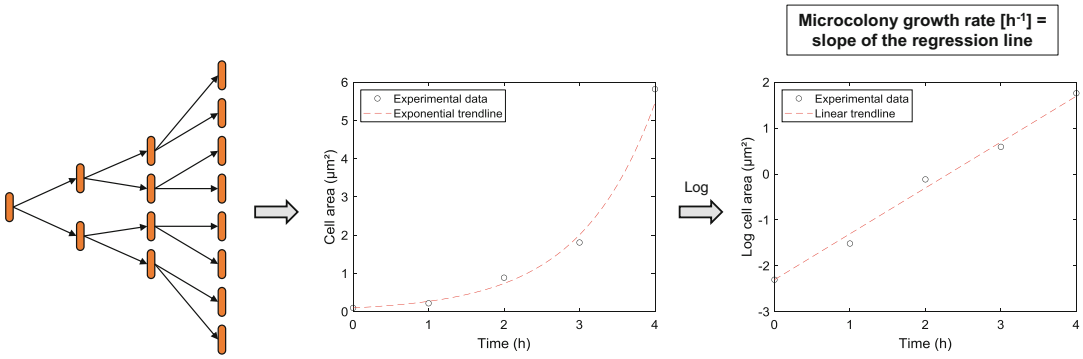

Fig. 3 Illustration of the calculation of the "Microcolony Growth Rate" Cumulative surface of all cells within a microcolony is monitored through time, yielding an exponential growth curve. Upon log-transformation of the data, a linear trend line is computed and the slope of it is determined and defined as the microcolony growth rate [h^{-1}]

microcolonies is similar to that of bacteria in flask cultures. However, single-cell heterogeneity also exists within a single microcolony, as discussed below.

3.2.5 Single-Cell Elongation Rate

The single-cell elongation rate is a property of a single cell at a given time-point as opposed to the microcolony growth rate, which is a property of a growing colony of cells issued from a mother cell (Figs. 3 and 4 illustrate the two metrics, respectively). The single-cell elongation rate is defined as the increment in cell surface per unit of time ($\mu m^2/h$). Its calculation should be performed on a short time-window, of the order of one generation time, to reflect instantaneous changes in the elongation rate at a given time point, typically at antibiotic treatment time. Briefly, cell area is plotted against time, giving rise to a typical saw-tooth profile (Fig. 4, left panel). The drop in cell area due to cell division events is compensated mathematically, giving rise to a linear increase in cell area against time (Fig. 4, right panel). Finally, the slope of this line is measured on a given time-frame (typically of the order of 1 generation time), defining the single-cell elongation rate (Fig. 4, right panel). This metric has been described and widely used in [7].

3.2.6 Persisters Growth Stage Identification

Exponentially growing bacteria can be differentiated from non-dividing bacteria based on single-cell elongation rate values at antibiotic treatment time as done in [7]. Growth status is an essential parameter due to the prevalent role of nongrowing subpopulations of cells in antibiotic persistence [19, 20]. Its relatively easy characterization at the single-cell level in the absence of any fluorescent reporters makes microfluidics a powerful tool for studying persisters physiology in their native state.

3.2.7 Fluorescent Biosensors

In addition to the growth stage and growth rate, specific physiological processes can be monitored by using fluorescent biosensors. Correlations or absence of correlations with survival are key to unravel molecular bases of persistence at the single-cell level. As an example, a fluorescent reporter of the *yefM-yoeB* TA system activation was previously used to demonstrate the absence of correlation between TA transcriptional activation and persistence at the single-cell level, since persister cells did not show increased fluorescence and since none of the highly fluorescent cells were able to resume growth after antibiotic treatment [21]. A similar strategy was used to correlate bacterial dormancy with concentration of the HokB toxic peptide as fluorescence of an mCherry-HokB fusion in *E. coli* cells was shown to correlate with the time before first cell division in microfluidics [22].

4 Notes

1. It is important for every element of the setup to be as closely as possible to the acquisition temperature to minimize drift during the experiment.

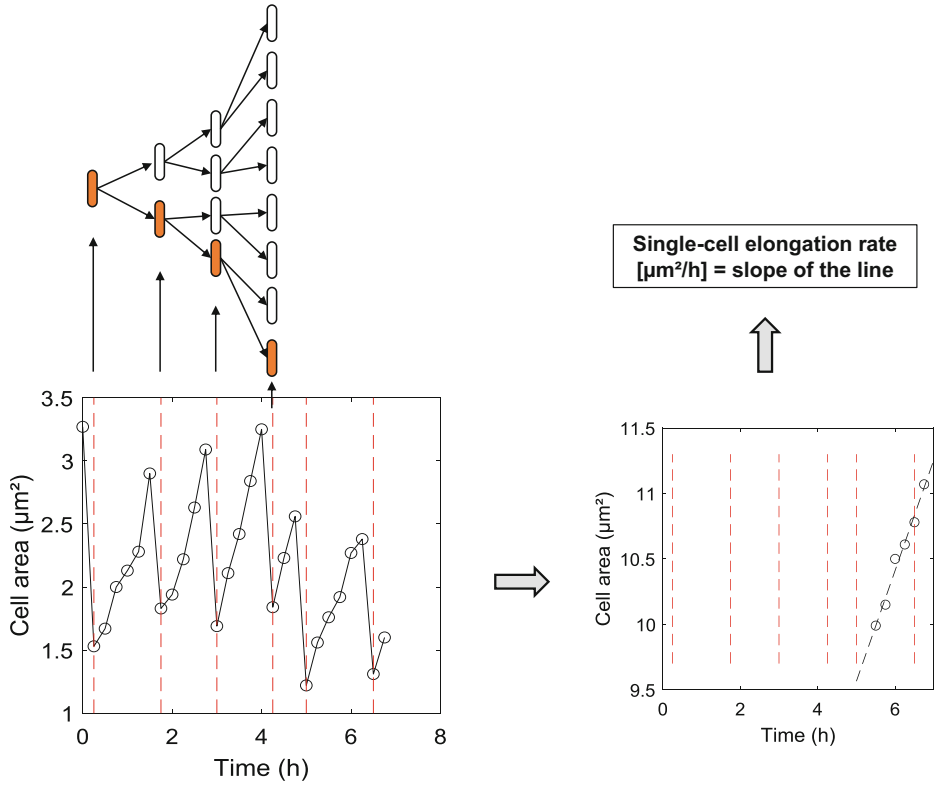

Fig. 4 Illustration of the calculation of the "Single-Cell Elongation Rate" Single-cell surface is monitored through time, yielding a typical saw-tooth profile as illustrated on the left panel. The drop in cell area due to cell division events is compensated mathematically, giving rise to a linear increase in cell area against time as shown in the right panel. Finally, the slope of this line is measured on a given time-frame (typically of the order of 1 generation time), defining the single-cell elongation rate (right panel) [$\mu m^2/h$]

2. A plate contains four microfluidic chambers. Each line of wells is connected to one chamber (A, B, C or D) (Fig. 1a). Each of these four chambers can be used to image different strains or to challenge these strains with different antibiotics. Each of the four chambers is marked with the letter of its well line (A–D) (Fig. 1b). Each chamber is subdivided in subchambers with different ceiling heights and marked with domino numerals (1–6) (Fig. 1b). *E. coli* cells are usually trapped in subchamber 5.
3. Since cells will be growing in the chamber before treatment, ensure that the seeding is not too dense (~a few cells per $1000\mu m^2$). If the seeding is not dense enough, repeat the Cell Loading Sequence.
4. Image acquisition, moving to the next field and autofocus all take a given amount of time, typically a few seconds. One should thus be careful not to image a number of fields that would take longer to acquire than the duration between two

snapshots (10–20 min). Photobleaching of fluorescent probes and phototoxicity of the illumination setup should be watched for as well.

5. It is good practice to monitor the first few time points to verify that images are acquired as they should be and that fields are properly focused.
6. Caution must be taken when growing cells in microfluidic devices as such model might not reflect the biology of bacteria in other growth conditions such as flask cultures or host environments. As a control, the frequency of survival and the growth rate should always be quantified and compared with those obtained in vitro or in vivo (flask cultures or host environment) to verify the validity of such a single-cell model. Major discrepancies might arise from (1) differences in aeration, (2) differences in nutrient availability, (3) the generation of chemical gradients due to improper perfusion of growth medium/antibiotics or crowded growth chambers, and (4) the adsorption of chemicals to the PDMS, a major component of most microfluidic devices. It is of good practice to monitor the speed and homogeneity of liquid perfusion in the microfluidic chamber using specific dyes such as fluorescein to ensure that medium replacement is efficiently observed in the desired time-window and to ensure that nutrient or antibiotic gradients are not present in the growth chamber. Such issues might generate critical experimental caveats by causing strong (and highly irrelevant) cell-to-cell heterogeneity.

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References

1. Balaban NQ, Helaine S, Lewis K et al (2019) Definitions and guidelines for research on antibiotic persistence. Nat Rev Microbiol 17:441–448
2. Balaban NQ, Merrin J, Chait R et al (2004) Bacterial persistence as a phenotypic switch. Science 305:1622–1625
3. Orman MA, Brynildsen MP (2013) Dormancy is not necessary or sufficient for bacterial persistence. Antimicrob Agents Chemother 57:3230–3239
4. Shah D, Zhang Z, Khodursky A et al (2006) Persisters: a distinct physiological state of *E. coli*. BMC Microbiol 6:53
5. Liu Z, Banaei N, Ren K (2017) Microfluidics for combating antimicrobial resistance. Trends Biotechnol 35:1129–1139
6. Wang P, Robert L, Pelletier J et al (2010) Robust growth of *Escherichia coli*. Curr Biol 20:1099–1103
7. Goormaghtigh F, Van Melderen L (2019) Single-cell imaging and characterization of

Escherichia coli persister cells to ofloxacin in exponential cultures. Sci Adv 5:eaav9462

8. Fraikin N, Goormaghtigh F, Van Melderen L (2020) Type II toxin-antitoxin systems: evolution and revolutions. J Bacteriol 202:e00763-19
9. Neidhardt FC, Bloch PL, Smith DF (1974) Culture medium for enterobacteria. J Bacteriol 119:736–747
10. Goormaghtigh F, Van Melderen L (2016) Optimized method for measuring persistence in *Escherichia coli* with improved reproducibility. Methods Mol Biol 1333:43–52
11. Ducret A, Quardokus EM, Brun YV (2016) MicrobeJ, a tool for high throughput bacterial cell detection and quantitative analysis. Nat Microbiol 1:16077
12. Stylianidou S, Brennan C, Nissen SB et al (2016) SuperSegger: robust image segmentation, analysis and lineage tracking of bacterial cells. Mol Microbiol 102:690–700
13. Lugagne JB, Lin H, Dunlop MJ (2020) DeLTA: automated cell segmentation, tracking, and lineage reconstruction using deep learning. PLoS Comput Biol 16:e1007673
14. Paintdakhi A, Parry B, Campos M et al (2016) Oufti: an integrated software package for high-accuracy, high-throughput quantitative microscopy analysis. Mol Microbiol 99:767–777
15. Sliusarenko O, Heinritz J, Emonet T et al (2011) High-throughput, subpixel precision analysis of bacterial morphogenesis and intracellular spatio-temporal dynamics. Mol Microbiol 80:612–627
16. Ollion J, Elez M, Robert L (2019) High-throughput detection and tracking of cells and intracellular spots in mother machine experiments. Nat Protoc 14:3144–3161
17. Young JW, Locke JC, Altinok A et al (2011) Measuring single-cell gene expression dynamics in bacteria using fluorescence time-lapse microscopy. Nat Protoc 7:80–88
18. van Raaphorst R, Kjos M, Veening JW (2020) BactMAP: an R package for integrating, analyzing and visualizing bacterial microscopy data. Mol Microbiol 113:297–308
19. Jõers A, Kaldalu N, Tenson T (2010) The frequency of persisters in *Escherichia coli* reflects the kinetics of awakening from dormancy. J Bacteriol 192:3379–3384
20. Gefen O, Fridman O, Ronin I et al (2014) Direct observation of single stationary-phase bacteria reveals a surprisingly long period of constant protein production activity. Proc Natl Acad Sci U S A 111:556–561
21. Goormaghtigh F, Fraikin N, Putrins M et al (2018) Reassessing the role of type II toxin-antitoxin systems in formation of *Escherichia coli* type II persister cells. mBio 9: e00640–e00618
22. Wilmaerts D, Dewachter L, De Loose PJ et al (2019) HokB monomerization and membrane repolarization control persister awakening. Mol Cell 75:1031–1042.e1034

Chapter 8

Microfluidics for Single-Cell Study of Antibiotic Tolerance and Persistence Induced by Nutrient Limitation

Stefany Moreno-Gámez, Alma Dal Co, Simon van Vliet, and Martin Ackermann

Abstract

Nutrient limitation is one of the most common triggers of antibiotic tolerance and persistence. Here, we present two microfluidic setups to study how spatial and temporal variation in nutrient availability lead to increased survival of bacteria to antibiotics. The first setup is designed to mimic the growth dynamics of bacteria in spatially structured populations (e.g., biofilms) and can be used to study how spatial gradients in nutrient availability, created by the collective metabolic activity of a population, increase antibiotic tolerance. The second setup captures the dynamics of feast-and-famine cycles that bacteria recurrently encounter in nature, and can be used to study how phenotypic heterogeneity in growth resumption after starvation increases survival of clonal bacterial populations. In both setups, the growth rates and metabolic activity of bacteria can be measured at the single-cell level. This is useful to build a mechanistic understanding of how spatiotemporal variation in nutrient availability triggers bacteria to enter phenotypic states that increase their tolerance to antibiotics.

Key words Antibiotic tolerance, Antibiotic persistence, Nutrient limitation, Single-cell measurements, Microfluidics, Biofilms, Feast-and-famine dynamics, Phenotypic heterogeneity

1 Introduction

Bacteria that are not genetically resistant to antibiotics can enter phenotypic states that allow them to survive antibiotic concentrations that would otherwise be lethal. Increasing evidence indicates that this phenomenon can be a major cause of treatment failure in the clinics [1–3] and can facilitate the evolution of antibiotic resistance in the long term [4–6].

Antibiotics often target cellular processes that operate when bacteria are metabolically active and dividing (e.g., the synthesis of the cell wall) [7, 8]. As a consequence, phenotypic states that allow bacteria to survive antibiotics are generally characterized by slower growth or complete growth arrest. Bacteria enter these

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states usually after being triggered by external abiotic or biotic factors. Most of these factors induce cellular stress and include starvation, acidity, and the host immune response [9–11]. These factors can cause a transient reduction in the growth rate in (a part of) the population, resulting in increased survival to antibiotic stress. While the term antibiotic tolerance refers to a population-wide increase in survival in the presence of antibiotics, persistence refers to the stochastic switch of only a subset of the population into protected phenotypic states [12, 13]. In practice, it can be hard to determine which scenario is at work especially when environments vary spatially. For simplicity, we will use the term tolerance in the remainder of this chapter, since bacterial cells that can survive antibiotics without being genetically resistant are called tolerant regardless of whether the underlying phenomenon is tolerance or persistence [12].

In nature, nutrient starvation is one of the most important factors leading to slow growing and nongrowing states in bacteria. In this chapter, we present two microfluidic setups to study how variation in nutrient availability in space or time can lead to increased tolerance to antibiotics (Fig. 1).

The first microfluidic setup allows to characterize how spatial variation in nutrient availability leads to increased antibiotic tolerance in clonal populations of bacteria [14, 15]. In this setup, bacteria grow as two-dimensional populations inside microfluidic chambers where nutrients diffuse into the population from a single side of the chamber (Fig. 2a, b). This setup mimics biofilm growth

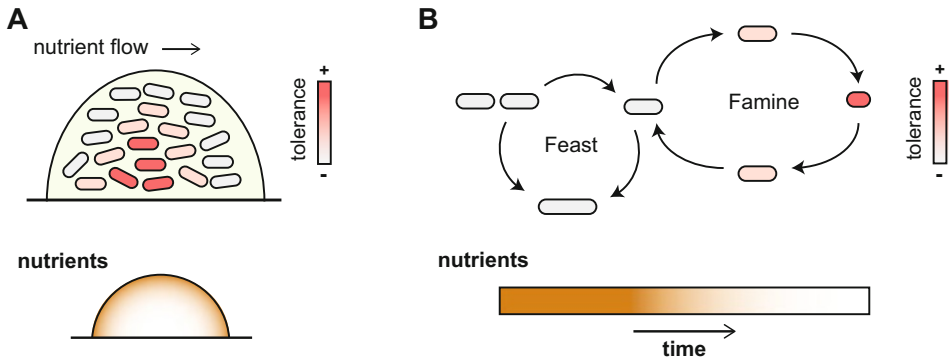

Fig. 1 Spatiotemporal regimes of variation in nutrient availability and antibiotic tolerance. In this chapter we present microfluidic setups to study antibiotic tolerance in two scenarios that often lead to nutrient limitation in nature. (**a**) In biofilms, spatial gradients in nutrient availability are common and limit the growth and metabolic activity of cells deep inside the biofilm. As a result, these cells grow more slowly, which increases their tolerance to antibiotics. (**b**) In nature, bacteria repeatedly switch between periods of feast and famine, spending a considerable amount of time in starved, nongrowing, states. These states offer protection to antibiotics because metabolic activities are minimal and cell growth is fully arrested. Some cells can remain in these nongrowing states for prolonged durations even after the bulk of the population has resumed growth, which increases antibiotic survival of clonal bacterial populations

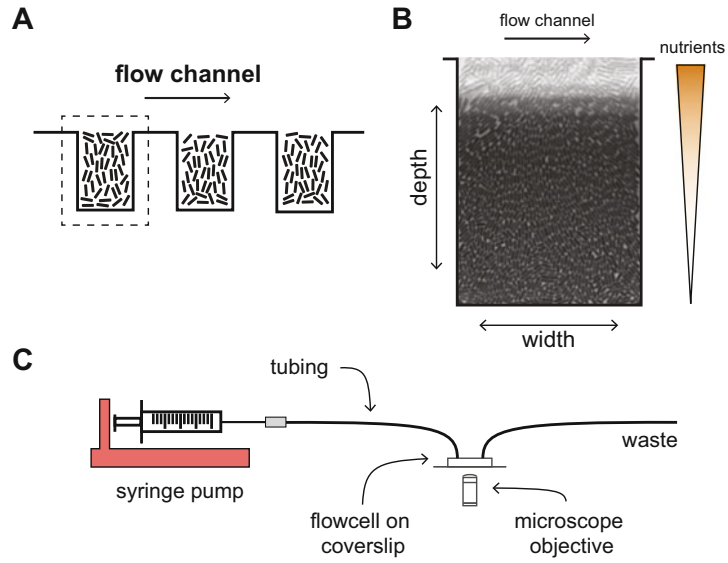

Fig. 2 Microfluidic setup to study the role of spatial heterogeneity in nutrient availability on antibiotic tolerance. (**a**) Scheme illustrating the "family" machine design of the microfluidic device. Each flow channel is ~2 cm long and there are several equally spaced growth chambers along the bottom and the top of the channel (only bottom side is shown). Inside a growth chamber (dotted line), bacteria grow in a densely packed two-dimensional layer. (**b**) Phase contrast image of a growth chamber from our experiments. Since nutrients diffuse into the chamber from only one side (at the top of figure), there is a spatial gradient in nutrient availability similar to those observed in biofilms. Thus, we can study the effect of phenotypic variation on antibiotic tolerance resulting from adaptation to different microenvironments (variation between cells at different depths) and phenotypic heterogeneity resulting from stochastic processes (variation between cells at same depth). The strength of the gradient can be manipulated by tuning the depth of the growth chambers or the concentration of the supplied nutrients. We used chambers that are 60 μm deep and 40 μm wide, since for these dimensions there is a strong gradient in the concentration of glucose for our experimental conditions [14]. The top of the image appears brighter because of imaging artifacts occurring at the chamber opening. These artifacts can be corrected when analyzing the images. (**c**) Overview of the entire setup. The growth medium is pumped into the flow cell using a syringe pump. The syringe is connected to the inlet port of the channel in the flow cell using the tubing. A short piece of thicker tubing (in gray) is used to connect the syringe needle to the tubing. Tubing also connects the outlet port of the channel to a waste container. The flow cell is placed under the microscope for continuous observation. The mold used in our experiments has eight parallel flow channels such that multiple conditions can be studied simultaneously

conditions (Fig. 1a). In fact, our chambers can be seen as a two-dimensional model (i.e., a slice) of a biofilm: Although biofilms have three-dimensional structures, nutrient availability in a biofilm primarily varies along a single direction away from the

surface where nutrients are present. In both natural biofilms and our growth chambers, cells take up and release nutrients and metabolites and thereby create spatial gradients in nutrient availability [15–17]. As a result, cells vary in their growth rate and metabolic activity depending on how far they are from the nutrient source. Under a constant flow of nutrients in the main channel of our microfluidic device, the cells in the growth chambers establish a stable gradient in nutrient availability and their growth rate decreases with the distance from the chamber opening. At this point, an antibiotic pulse can be applied to the population to study how variation in growth rate and metabolic activity increases tolerance to antibiotics in the population.

The second microfluidic setup allows to characterize how variation in nutrient availability over time leads to increased tolerance to antibiotics. In particular, our setup captures the temporal dynamics of nutrient availability characteristic of feast-and-famine regimes (Fig. 1b). These regimes are recurrently encountered by bacteria in nature and are likely to be relevant in environments like the human gut. In our setup, cells grow in a microfluidic device that is connected to a batch culture (Fig. 3). Cells in the batch culture are inoculated at a low density and gradually deplete the available nutrients until they become starved. Since bacteria in the microfluidic device are coupled to the batch culture, they will follow the same transition: They first grow exponentially but then, as nutrients are depleted, their growth rate decreases until they reach full growth arrest. Bacteria in the microfluidic device remain starved for as long as they are connected to the batch culture with depleted nutrients. Then, they can be switched to fresh media and exposed to an antibiotic pulse either at the same time that nutrients are provided [9] or sometime after this switch [18]. In this way, one can study how the timing of growth resumption of a cell affects the probability that the cell survives antibiotic exposure after starvation.

These microfluidic setups have two features that are important to study the role of nutrient limitation on antibiotic tolerance. First, they allow to closely mimic the conditions that lead to nutrient limitation in natural environments. In particular, in both setups nutrient availability changes as a result of the collective metabolic activity of the population, which captures the fundamental role that bacteria play in driving nutrient limitation in their own environment. Moreover, these setups are useful to disentangle different factors that may lead to antibiotic tolerance in nature. For instance, in the spatial setup one can study how antibiotic tolerance varies with the size of a biofilm by changing the dimensions of the microfluidic chambers. On the other hand, in the temporal setup, one can study how the timing and duration of antibiotic exposure relative to periods of feast and famine affects the fraction of tolerant bacteria. Both setups can also be combined to study natural scenarios where spatial as well as temporal variation in nutrient availability are relevant to understand why bacteria become tolerant to

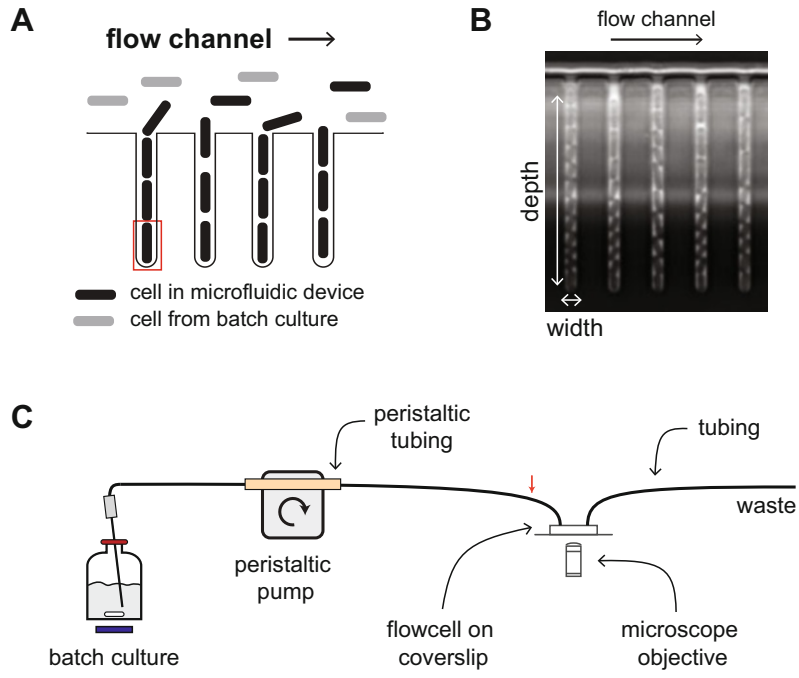

Fig. 3 Microfluidic setup to study the role of temporal heterogeneity in nutrient availability on antibiotic tolerance. (**a**) Scheme illustrating the "mother" machine design of the microfluidic device. Each flow channel is ~2 cm long and there are several equally spaced growth chambers along the bottom and the top of the channel (only bottom side is shown). In order to starve bacteria in the microfluidic device, the device is connected to a batch culture. As a result, there is a continuous flow of cells from the culture passing by the main flow channel of the device. We focus our analysis only on the mother cells (red box) because these cells will always stay in the growth chambers. Also, since this setup is used to quantify phenotypic heterogeneity, using only the mother cells ensures that all cells that are analyzed are at the same distance from the opening of the growth chamber and thus experience practically identical microenvironments. (**b**) Phase contrast image of the growth chambers in our device. Chambers are 25 μm deep and 1.2–1.6 μm wide. Depending on the growth conditions, it might be advisable to modify the dimensions of the growth chambers (*see* **Note 5**). (**c**) Overview of the first phase of the setup where cells in the microfluidic device transition from exponential to stationary phase. The batch culture used to starve cells in the microfluidic device is grown in a serum flask and continuously aerated using a magnetic stirrer (in blue) and a stir bar. The medium is drawn from the flask into the flow cell using a hubless needle that is connected to a peristaltic pump. Depending on the dimensions of the tubing and the needle, thick tubing (in gray) might be needed to make this connection. Once bacteria enter starvation they will remain in this phase for as long as the device is connected to the batch culture. In the second phase of our setup, starvation ends and bacteria are switched to fresh medium and exposed to the antibiotic pulse. This switch is done at a fixed location close to the inlet of the flow cell (orange arrow) and media is pumped into the flow cell using syringe pumps in the same manner as in the spatial setup (Fig. 2c)

antibiotics. Second, these setups are designed to characterize the effect of nutrient limitation on antibiotic tolerance at the single-cell level. Investigating how the phenotype of a cell affects its chance to survive antibiotic exposure can provide a better understanding of the molecular mechanisms behind increased survival to antibiotics. For example, one can track cell lineages and correlate the cell size, growth rate, or division state with the probability that a cell survives antibiotic exposure. Then, one can use fluorescent reporters to integrate the previous data with information on the activity of particular metabolic or physiological functions that might affect antibiotic tolerance (e.g., synthesis of efflux pumps or toxin-antitoxin systems).

2 Materials

2.1 Bacterial Strains and Growth Media

1. Bacterial strain of interest. We used *Escherichia coli* MG1655 to develop and standardize both experimental setups. However, these setups can be adapted to study the effect of nutrient limitation on antibiotic tolerance in other strains of *E. coli* or other bacterial species (*see* **Notes 1–3**).
2. Growth medium of interest. In order to precisely control nutrient concentrations we used M9 minimal medium supplemented with 0.01% Tween 20 and glucose at concentrations ranging from 800 μM to 1.11 mM. Other growth media can be used if needed and a surfactant like Tween can be added to the medium to prevent biofilm formation (*see* **Note 2**).

2.2 Preparation of Microfluidics Device

1. Mold with the appropriate design to produce microfluidic flow cells for each setup (*see* **Note 1**). The designs presented in this chapter are available at Metafluidics (*see* **Note 4**). Molds can be fabricated in a cleanroom facility or ordered commercially. A brief description of the designs for each setup is given below.
   (a) Family machine design for spatial heterogeneity setup. In this design, there is a main flow channel that is ~2 cm long, 100 μm wide and 23 μm high with growth chambers attached on both sides (Fig. 2a). Growth chambers are 0.76 μm high, 40 μm wide, and 30 μm or 60 μm deep. The strength of the nutrient gradient can be controlled by varying the depth of the growth chamber or the concentration of the nutrient [14].
   (b) Mother machine design for temporal heterogeneity setup. In this design, there is a main flow channel that is ~2 cm long, 200 μm wide and 21 μm high with growth chambers on both sides. These chambers are 0.93 μm high, 25 μm deep, and have a width ranging from 1.2 to 1.6 μm, so they are much narrower than the chambers of the family design (Fig. 3a) (*see* **Note 5**).

2. Curing agent and Polydimethylsiloxane (PDMS, Sylgard 184 Silicone Elastomer Kit, Dow Corning).
3. Vacuum desiccator with pump.
4. Plasma surface treatment system. For our experiments we used a Harrick Plasma PDC-32G-2 Plasma Cleaner.
5. Robins Instrument True-Cut Disposable Biopsy Punch 0.5 mm (*see* **Note 6**).
6. Scalpel.
7. Nr. 1.5 coverslips that are as large, or larger, than the microfluidic device. We used Menzel-Gläser 50 mm round coverslips.
8. Hot plate.
9. Ethanol and/or isopropanol (IPA).

2.3 Cell Observation and Microfluidic Experiments

1. Inverted microscope capable of phase contrast and epifluorescence imaging, equipped with a temperature-controlled incubator. A high resolution objective (e.g., 100× oil objective) and autofocus system are highly recommended.
2. Microfluidic tubing with a diameter that provides a tight fitting to the inlet and outlet ports of the microfluidic flow cell. Since media will be stored and pumped from a syringe, a thicker tubing might also be necessary for connecting the tubing to the tip of the syringe needle (*see* **Note 7**). The reference of the tubing used in our experiments is Teflon, inner diameter 0.3 mm, outer diameter 0.76 mm (Fisher Scientific). For the thicker tubing the reference is Microbore Tygon S54HL, inner diameter 0.76 mm, outer diameter 2.29 mm (Fisher Scientific). For the needles the reference is Sterican needles 20G, 0.9 mm × 70 mm (Braun).
3. Syringes and high precision programmable syringe pump. A multichannel pump is recommended so multiple channels from the flow cell can be used simultaneously. We used pumps from New Era Pump Systems but other pumps (e.g., from ElveFlow) are also compatible with our setups.

2.4 Additional Equipment for Setup with Temporal Heterogeneity in Nutrient Availability

1. Peristaltic pump (IPC-N24 from ISMATEC) and PharMed Ismaprene tubing with inner diameter 0.25 mm, outer diameter 2.07 mm and wall thickness 0.91 mm (VWR) to connect and pump media from the batch culture into the microfluidic device (*see* **Note 8**).
2. Serum flasks with rubber lids. We use flasks with a volume of 250 mL. This can be adjusted depending on the size of the incubation box.
3. Micro magnetic stirrers with stir bars to grow and agitate the batch cultures.

4. Hubless needles to draw media from the batch cultures.
5. If it is not possible to place the serum flasks inside the incubation box from the microscope or if the temperature inside the box cannot be reliably maintained away from the objectives (check this using an infrared thermometer), a separate incubator with a small entry port for tubing and cables for the magnetic stirrers is also needed. This incubator would have to be placed next to the microscope.

3 Methods

3.1 Preparation of Microfluidics Devices

Next we describe the protocol to prepare a microfluidic device. Although the molds differ for both setups, this protocol is the same in both cases.

1. Mix PDMS thoroughly with the curing agent in a 1:10 ratio. A ratio of 1.5:10 can be used to make the PDMS stiffer which can help prevent the collapse of chambers in the family machine design.
2. Pour the mix onto the mold and place into the desiccator until all air bubbles have been removed (approximately 30 min).
3. Place the mold in the oven and cure PDMS at 80 °C for 1 h.
4. Cut out the microfluidic flow cell with a scalpel and make ports for the inlet and outlet of each channel with a hole puncher. It is essential to be careful when cutting to prevent permanent damage to the mold. For silicon-based molds make sure to minimize the amount of pressure applied on the mold to prevent breaking.
5. Before binding, wash the glass coverslip with ethanol or IPA. Then, clean the surface of the flow cell that has the imprinted features using scotch tape and rinse it with water and IPA if needed. Check that water flows through all the ports that were punched in the previous step. Finally, dry the coverslip and flow cell thoroughly with pressurized air.
6. Bind the flow cell to the glass coverslip using plasma treatment. Place the coverslip and the flow cell (with the side that has the features upward) inside the plasma surface treatment system. Then, switch on the vacuum pump and treat both surfaces with oxygen plasma. We used a Harrick Plasma PDC-32G-2 Plasma Cleaner, at a processing pressure of 2 mbar, with 30–60 s of high power. With other plasma treatment devices the treatment time and power need to be optimized. With the help of a pair of tweezers, put the activated side of the flow cell on top of the activated side of the coverslip and visually confirm that binding takes place. If needed gently tap on the flow cell with the

tweezers to facilitate binding (avoid tapping directly on top of the growth chambers). Afterward, place the bonded device on top of a heated hot plate at 100 °C for 1 min. These steps might require some optimization to prevent unbinding of the flow cell from the coverslip (*see* **Note 9**).

3.2 Setup with Spatial Heterogeneity in Nutrient Availability

First, bacterial cells are loaded in the microfluidic device. Subsequently, they are grown till the growth chambers are completely full after which the nutrient gradients can be established. Afterward, bacteria are exposed to antibiotics for a fixed window of time before finally allowing them to recover in a rich growth media to assess cell survival. These steps are explained in more detail below.

1. One day before starting the experiment, start an overnight culture for loading the microfluidic device. We started this culture from a single colony picked from an LB agar plate so the cells loaded in the device can be assumed to be genetically identical.
2. To start loading the day of the experiment, first prewet the microfluidic device with culture medium using a pipette. Then, make sure to remove as much liquid as possible by pushing air into the flow channel using an empty pipette.
3. Centrifuge 1 mL of the overnight culture and resuspend the pellet in about 10 μL of the same growth medium to concentrate the cells (*see* **Note 10**).
4. Pipet ~1 μL of concentrated cells into the flow cell from the outlet of the channel. If possible, avoid that the liquid reaches all the way to the inlet.
5. Place the microfluidic device under the microscope. To load the cells inside the growth chambers, connect an empty syringe to the outlet port of the channel and bring the concentrated cell solution back and forth using a small air bubble. In this way the growth chambers will dry up sucking inside the cells in the concentrated solution (*see* **Note 11**). Note that only one cell is necessary within each growth chamber for the chamber to be loaded.
6. Fill a syringe with a medium that allows for rapid growth of all cells in the chamber (also the ones furthest away from the chamber opening) to quickly create a dense monolayer of cells in the chambers (*see* **Note 12**). In our experiments we used M9 media supplemented with 10 mM glucose.
7. Connect the tubing to the syringe needle using a short piece of thick tubing before connecting it to the inlet of the loaded channel (Fig. 2c, *see* **Note 13**). Make sure that the tubing is full of media without any air bubbles before making the latter connection.

8. Connect tubing to the outlet of the channel to collect the media flowing out of the flow cell in a waste container.
9. Switch the syringe pump on and let the cells grow until they fill up the growth chambers. For the strain and growth conditions we used this takes ~18 h. Set the flow rate to the final rate that will be used in the experiment (*see* **Note 14**). In our experiments we used 0.5 mL/h.
10. Start time-lapse microscopy (*see* **Note 15**). The imaging protocol should be compatible with the planned data analysis and early testing of the data analysis pipeline is recommended, such that the imaging protocol can be adjusted if needed. For example, if cell tracking is planned, phase contrast images should be taken often, as automated tracking software typically requires small movement of cells between subsequent frames (we imaged every 1.75 min). A good reference is that one should be able to track cells by eye in a recorded movie without too much difficulty. Fluorescence images to measure the expression of reporter genes can be taken less often.
11. Once the growth chambers are full, switch to a medium with a low nutrient concentration such that a spatial gradient in nutrient availability arises within each growth chamber. In our experiments we used M9 media supplemented with 800 μM of glucose (*see* **Note 16**). This switch is done by disconnecting the tubing from the first syringe and sliding it on the syringe that contains the new media. Make sure that no air bubbles are introduced and that the thick tubing is not damaged during the switch (*see* **Note 17**).
12. Wait for a few hours for the system to reach a steady state. In our experiments this took ~4 h.
13. Apply the antibiotic pulse by switching the cells to the same low nutrient medium supplemented with antibiotics for a fixed window of time (*see* **Note 18**). In our experiments we used streptomycin at a concentration of 50 μg/mL for 3 h. The switch is done as described in **step 11**.
14. After the window of antibiotic exposure, switch bacteria to a medium with high nutrient concentration to determine which cells survived antibiotics. A high nutrient concentration is essential to make sure that all surviving cells have access to nutrients, irrespective of how far away they are from the chamber opening. Bacteria should remain in this condition for a long period of time to make sure that surviving cells have enough time to resume growth. In our experiments we used M9 media supplemented with 10 mM glucose for 35 h.
15. To further confirm which cells survived the antibiotic pulse, a fluorescent dye can be used in this step to quantify the membrane potential of the cells. In particular, this dye could allow

one to gain information about cells that did not resume growth after the antibiotic pulse ended. Apply the fluorescent dye by switching the cells to a syringe with medium containing the dye. Then, use fluorescence imaging to determine the intracellular concentration of the dye in all cells in the population.

3.3 Setup with Temporal Heterogeneity in Nutrient Availability

First, cells are loaded in the microfluidic device which is connected to a flask with medium so they grow for a few hours and get adjusted to the device. Second, the flask that is connected to the device is inoculated to start the batch culture. Cells in the batch culture will start dividing and eventually deplete the nutrients at which point cells in the flow cell enter starvation. The duration of starvation is set by how long the microfluidic device remains connected to the culture with depleted nutrients. Third, bacteria in the microfluidic device are switched to fresh media. At this point the antibiotic pulse can be applied together with the switch to fresh media to reproduce batch setups like the one presented by Fridman et al [9]. Alternatively, cells can first be grown on fresh media before switching to antibiotics as done by Moreno-Gámez et al [18]. Finally, after the antibiotic pulse, bacteria are switched to fresh media to determine which cells managed to survive the pulse and further characterize the dynamics of growth resumption from starvation. These steps are explained in more detail below.

1. One day before starting the experiment, start an overnight culture for loading. We started this culture from a single colony picked from an LB agar plate so the cells loaded in the device can be assumed to be genetically identical.
2. Sterilize the serum flask in which the batch culture will be grown. The flask should have a stir bar inside and the lid should be punched with a hubless needle that goes all the way to the bottom of the flask. This needle will be used to draw the media into the flow cell.
3. On the day of the experiment, start by connecting both ends of the peristaltic tubing to the microfluidic tubing. Use tape to secure these connections. One end of the tubing will be connected to the flask with the batch culture and the other end will go into the flow cell (Fig. 3c).
4. Add the batch growth medium to the sterile serum flask (*see* **Note 19**). Then, connect the part of the hubless needle that sticks out from the serum flask to one end of the tubing prepared in the previous step. Depending on the size of the needle a short piece of thick tubing can be needed for this connection (Fig. 3c).
5. Bring the serum flask with the attached tubing to the incubator and place the peristaltic tubing in the peristaltic pump (*see* **Note 20**). Place the flask on top of the magnetic stirrer (*see* **Note 21**)

and switch the pump on at a low rate to have liquid ready to come out at the end of the tubing that will go into the flow cell. This also helps prevent contamination.

6. Load the cells as described in Sect. 3.2, **steps 2–5**. Due to the narrower chambers, loading the mother machine is harder than loading the family machine so it might be necessary to move the air bubble multiple times along the flow channel to improve loading.
7. Once the cells are loaded, connect the serum flask to the flow cell at the inlet (*see* **Note 13**). Then, connect a piece of tubing to the outlet of the channel to collect the medium flowing out in a waste bottle. Set the flow rate to the final rate that will be used for the experiment (in our experiments we used 0.5 mL/h, *see* **Note 14**), and let the cells grow for a few hours so they acclimate to the flow cell.
8. Start time-lapse microscopy taking images regularly in order to estimate growth rates at the single-cell level (*see* **Note 15**). The imaging protocol should be compatible with the planned data analysis and early testing of the data analysis pipeline is recommended, such that the imaging protocol can be adjusted if needed (*see* Sect. 3.2, **step 10**). By recording how cells enter stationary phase one can study whether the survival of cells during starvation as well as their dynamics of growth resumption depend on their growth dynamics before starvation.
9. Inoculate bacteria from an overnight culture into the serum flask using a pipette and switch on the magnetic stirrer. Always use the same dilution factor to keep the length of the exponential phase constant. Since the lid of the serum flask has to be lifted to inoculate the cells, switch off the peristaltic pump temporarily to avoid drawing up air into the flow cell.
10. Once cells have entered stationary phase, leave the flow cell connected to the batch culture for the duration of starvation. How long cells spend in starvation will determine their lag time distribution once fresh resources are supplied and thus their tolerance to antibiotics [18]. One can considerably decrease the frequency of time-lapse imaging in this step since cell growth is fully arrested.
11. To end starvation, fill a syringe with the medium for growth resumption (*see* **Note 12**), place it on a syringe pump and connect the microfluidic tubing to the syringe needle using a piece of short thick tubing. One can choose to add antibiotics to the medium already at this step of the protocol, which would emulate previous batch setups to study antibiotic tolerance [9]. If so, proceed to **step 13** after completing this step. To do the switch, turn off the peristaltic pump and cut the tubing

at a fixed short distance (e.g., 20 cm) from the inlet to disconnect the flow cell from the batch culture (Fig. 3c). Then, connect the tubing coming from the syringe to the tubing going into the inlet of the flow cell using a piece of thick tubing. As always, avoid introducing air bubbles during the switching of the tubing (*see* **Note 17**). A pinch valve can be used in this step to simplify the switch from the batch culture to the fresh medium [21].

12. If bacteria were not exposed to antibiotics in the previous step, prepare a syringe with the same medium used for growth resumption supplemented with antibiotics. Then, switch bacteria to this medium for a fixed window of time. This switch can be done at the same location up from the inlet where the connection was done in the previous step or at the level of the syringes as in Subheading 3.2, **step 11** (*see* **Note 18**). Depending on the experimental question one can vary both the time at which the pulse is applied as well as the duration of the pulse. In our experiments we applied the antibiotic pulse 8.6 h after the switch to fresh medium and the pulse lasted 80 min [18]. For the pulse, we used ampicillin at a concentration of 100 μg/mL.
13. After the window of antibiotic exposure, switch the bacteria back to a fresh medium to determine which cells survived the antibiotics and to further quantify the dynamics of growth resumption at the single-cell level. Bacteria should remain in this condition for a long period of time to make sure that cells with prolonged lag times will have enough time to resume growth. In our experiments we kept observing cells for 40 h after the antibiotic pulse had ended.
14. To further confirm which cells survived the antibiotic pulse, apply a fluorescent dye to measure the membrane potential of the cells (*see* Subheading 3.2, **step 15**).

4 Notes

1. In order to adapt the setups to study other bacterial species, the dimensions of the molds and in particular their height might have to be adjusted to the expected cell size under the planned growth conditions. It is critical that the height of the growth chambers matches closely with the expected cell size. Chambers that are too low lead to growth defects [19] and chambers that are too high can lead to loss of cells due to washout events and/or growth of cells in multiple layers.

2. Some bacterial species are more prone to form biofilms that can clog the device or tubing. There are several solutions to reduce biofilm formation. First, surfactants (e.g., Tween) and bovine serum albumin (BSA) can be added to the media at low concentration. The low concentration is important to avoid that bacteria utilize these compounds as a carbon source. Second, the microfluidic device can be pretreated with compounds like BSA to passivate surfaces [20]. Third, the flow rate during the experiment can be increased. Note however that a very high flow rate can increase washout of the cells from the device. Fourth, as most cell attachment happens during loading, one can load with early exponential-phase cells (which are generally less sticky) and reduce the concentration of the cell suspension (e.g., by 10×). Finally, one can use strains with loss of function mutations for genes involved in biofilm formation.
3. It is advisable to use bacterial strains with low or no motility. Otherwise, bacterial cells might swim out of the devices.
4. Family machine design: https://metafluidics.org/devices/family-machine-to-study-spatial-gradients-in-2d-bacterial-populations/. Mother machine design: https://metafluidics.org/devices/mother-machine-to-study-feast-and-famine-dynamics/.
5. If using a mother machine device with the same dimensions as ours, make sure to use a sugar concentration that is high enough to avoid strong spatial gradients along the growth chambers. Alternatively, reduce the depth of the chambers to lengths of 10–15 μm. Washout of cells increases in shorter channels so it is important to do some testing before settling down on a particular depth. Further optimization of other dimensions of the device might be necessary to avoid clogging due to the continuous flow of cells from the batch culture [21]. This can be a problem especially if the batch culture reaches a high density.
6. In order to prevent leakage, it is essential that the size of the hole punchers matches the size of the tubing so there is tight fit at the inlet and outlet ports of the flow cell. The 0.5 mm punchers work well with tubing that has the dimensions specified in Subheading 2.2.
7. The tubing can be connected directly to the syringe needle if tubing and needles with different dimensions are used. We did not use these for our experiments but they are often used in similar microfluidic setups [20]. The references are PTFE Tubing, 0.56 mm inner Diameter × 1.07 mm outer Diameter (Adtech) and Microlance 3, 0.55 × 25 mm syringe needles. These work well together with a 0.75 mm hole puncher.

8. Since bacteria from the batch culture are being flown into the microfluidic device, there might be biofilm formation in the peristaltic tubing. If none of the solutions proposed in **Note 2** solves this problem, try using a different type of peristaltic tubing than the one suggested here.
9. There are several modifications of the protocol that can be implemented to avoid unbinding of the flow cell from the coverslip. These include increasing the power or the length of plasma treatment, cleaning flow cells and coverslip more thoroughly with water and a solvent (e.g., IPA) and baking flow cells after binding. Another possibility is to reduce the flow rate during the experiment. However, note that a very low flow rate can increase biofilm formation.
10. In case of issues with loading the device, use a growth medium with a high carbon concentration to get a more concentrated cell solution for loading or with a composition that would make cells smaller during stationary phase. Alternatively, try loading cells that are in exponential phase instead of stationary phase.
11. It is essential to minimize the time in which cells are exposed to the air bubble to prevent cell damage or death.
12. Calculate the volume of medium needed to fill a syringe based on the flow rate that will be used and the total duration of the experiment. Always add some extra volume since liquid is often lost when setting up the experiment (e.g., when placing the syringes in the pump). It is very important to eliminate air bubbles from the medium when filling up the syringes.
13. To connect tubing to the inlet or the outlet of a channel always place the microfluidic device on a flat surface, or position an in-focus oil objective directly below the insertion side. Otherwise it is easy to break the glass coverslip. Also, make sure that the tubing goes deep into the ports.
14. When choosing the flow rate for an experiment keep in mind the trade-off underlying this choice. Low flow rates decrease the chance that the flow cell unbinds from the coverslip and prevent washouts. Also, when the flow rate is low less medium is spent during an experiment. However, low flow rates will increase the chance of biofilm formation in the tubing and in the main channel of the device.
15. Make sure to firmly secure all the parts of the setup with tape such that everything stays in place during time-lapse imaging. This includes the tubing and the slide holder with the microfluidic device.

16. Use pilot experiments to establish the relationship between the nutrient concentration and the gradient depth and to establish the required time for a stable gradient to form.
17. If an air bubble gets into the tubing when doing a media switch, make sure that the bubble rapidly passes through the microfluidic device by temporarily increasing the flow rate. Alternatively, if the tubing is long enough, one can cut off the part that contains the air bubble and reattach it to the syringe.
18. There is a delay between the time at which the media is switched at the syringe and the time at which cells in the flow cell are exposed to the new media. The duration of this delay depends on the length of the tubing and the flow rate and is essential to take into account when characterizing the response of cells to an antibiotic pulse. This delay can be measured in a control experiment where a switch is done between a normal medium and a medium containing a fluorescent dye or fluorescent beads. A fluorescent dye or fluorescent beads can also be added to the medium containing the antibiotics to have an internal control, as long as they do not interfere with the fluorescent reporters that are used.
19. Calculate the volume of medium that needs to be added to the serum flask based on the flow rate and the total duration of the periods of exponential growth and starvation. To avoid drawing up air bubbles into the microfluidic device, a 250 mL flask should always have a volume of ~20 mL left after starvation is over. Once the medium is added, make sure that the tip of the hubless needle reaches all the way to the bottom of the flask.
20. If possible, place the peristaltic pump inside the incubation box to keep the cells at a constant temperature while they travel to the flow cell. Otherwise, maximize the amount of tubing inside the incubation box and measure the temperature of the tubing outside to make sure deviations from the growth temperature are minor.
21. Since the culture inside the flask will be agitated, make sure that the flask will not slip from the top of the magnetic stirrer. Putting a piece of parafilm underneath the flask is useful to increase friction. Alternatively, consider building a supportive structure to keep the flask in place. Once the magnetic stirrer is switched on, make sure that the stir bar does not bump into the needle.

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References

1. Vulin C, Leimer N, Huemer M et al (2018) Prolonged bacterial lag time results in small colony variants that represent a sub-population of persisters. Nat Commun 9:4074
2. Fauvart M, De Groote VN, Michiels J (2011) Role of persister cells in chronic infections: clinical relevance and perspectives on anti-persister therapies. J Med Microbiol 60:699–709
3. Mulvey MA, Schilling JD, Hultgren SJ (2001) Establishment of a persistent *Escherichia coli* reservoir during the acute phase of a bladder infection. Infect Immun 69:4572–4579
4. Levin-Reisman I, Ronin I, Gefen O et al (2017) Antibiotic tolerance facilitates the evolution of resistance. Science 355:826–830
5. Fisher RA, Gollan B, Helaine S (2017) Persistent bacterial infections and persister cells. Nat Rev Microbiol 15:453–464
6. Bakkeren E, Diard D, Hardt W-D (2020) Evolutionary causes and consequences of bacterial antibiotic persistence. Nat Rev Microbiol 18:479–490
7. Cho H, Uehara T, Bernhardt TG (2014) Beta-lactam antibiotics induce a lethal malfunctioning of the bacterial cell wall synthesis machinery. Cell 159:1300–1311
8. Kohanski MA, Dwyer DJ, Collins JJ (2010) How antibiotics kill bacteria: from targets to networks. Nat Rev Microbiol 8:423–435
9. Fridman O, Goldberg A, Ronin I et al (2014) Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. Nature 513:418–421
10. Manina G, Dhar N, McKinney JD (2015) Stress and host immunity amplify *Mycobacterium tuberculosis* phenotypic heterogeneity and induce nongrowing metabolically active forms. Cell Host Microbe 17:32–46
11. Booth IR (2002) Stress and the single cell: intrapopulation diversity is a mechanism to ensure survival upon exposure to stress. Int J Food Microbiol 78:19–30
12. Balaban NQ, Helaine S, Lewis K et al (2019) Definitions and guidelines for research on antibiotic persistence. Nat Rev Microbiol 17:441–448
13. Brauner A, Fridman O, Gefen O et al (2016) Distinguishing between resistance, tolerance and persistence to antibiotic treatment. Nat Rev Microbiol 14:320–330
14. Dal Co A, van Vliet S, Ackermann M (2019) Emergent microscale gradients give rise to metabolic cross-feeding and antibiotic tolerance in clonal bacterial populations. Philos Trans R Soc Lond Ser B Biol Sci 374:20190080
15. Dal Co A, Ackermann M, van Vliet S (2019) Metabolic activity affects the response of single cells to a nutrient switch in structured populations. J R Soc Interface 16:20190182
16. Stewart PS, Franklin MJ (2008) Physiological heterogeneity in biofilms. Nat Rev Microbiol 6:199–210
17. Flemming H-C, Wingender J, Szewzyk U et al (2016) Biofilms: an emergent form of bacterial life. Nat Rev Microbiol 14:563–575
18. Moreno-Gámez S, Kiviet DJ, Vulin C et al (2020) Wide lag time distributions break a trade-off between reproduction and survival in bacteria. Proc Natl Acad Sci U S A 117:18729–18736
19. Yang D, Jennings AD, Borrego E et al (2018) Analysis of factors limiting bacterial growth in

PDMS mother machine devices. Front Microbiol 9:871

20. Kaiser M, Jug F, Julou T et al (2018) Monitoring single-cell gene regulation under dynamically controllable conditions with integrated microfluidics and software. Nat Commun 9:212

21. Bakshi S, Leoncini E, Baker C et al (2020) Dynamic regulation of growth and physiology of microbes under complex changing conditions. https://doi.org/10.1101/2020.03.27.006403

Chapter 9

Counting Chromosomes in Individual Bacteria to Quantify Their Impacts on Persistence

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Abstract

Persisters are phenotypic variants within bacterial populations that tolerate antibiotic treatments considerably better than the majority of cells. A phenotypic quality that varies within bacterial populations is the chromosome number of individual cells. One, two, four, or more chromosomes per cell have been observed previously, and the impact of genome copy number can range from gene dosage effects to an inability to perform specific DNA repair functions, such as homologous recombination. We hypothesize that chromosome abundance is an underappreciated phenotypic variable that could impact persistence to antibiotics. Here, we describe methodologies to segregate bacterial populations based on chromosome number, assess the purity of those subpopulations, and suggest assays that could be used to quantify the impacts of genome abundance on persistence.

Keywords Persistence, Heterotolerance, Chromosomes, FACS, Homologous recombination, Ploidy

1 Introduction

Bacterial persisters are phenotypic variants within bacterial populations that tolerate antibiotic treatment better than their surrounding kin [1]. Persistence is a form of heterotolerance, which is why biphasic kill curves are used to detect their presence: The majority of antibiotic-susceptible cells die rapidly (first characteristic death rate), whereas persisters, which constitute minority subpopulations, exhibit slower rates of killing (second characteristic death rate) [1, 2]. Importantly, if persisters were harvested and the assay was conducted again, a comparable kill curve would be observed, which testifies to the phenotypic nature of the persistence phenomenon [3, 4]. The molecular composition of persisters in comparison to cells that die rapidly is a topic of immense research interest [2, 5, 6], and the underlying question is, what makes persisters special?

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Several recent reviews have summarized molecular features that increase or decrease the likelihood a bacterial cell will give rise to a persister [5–8]. However, one phenotypic variable that has largely been ignored is chromosome copy number. Exponentially growing bacterial cells, for example, can have up to 16 full- and partially replicated chromosomes due to overlapping cell cycles [9–12], whereas stationary-phase bacteria tend to have an integer number of fully replicated chromosomes due to the lack of new rounds of replication initiation [4, 10]. This variation in ploidy status has been shown to impact microbial physiology in different ways [13, 14]. Haploid *Lactococcus lactis*, for example, is more susceptible to ultraviolet radiation than its diploid counterpart [15]. Similarly, *Deinococcus radiodurans* and *Thermus thermophilus* are polyploid organisms, whose chromosomal content may enable them to better survive radiation and thermal stresses, respectively [16, 17]. Along these lines, chromosomal content may impact DNA repair mechanisms including homologous recombination, which requires the presence of an undamaged sister chromosome for accurate repair of damaged DNA [18]. Furthermore, gene dosage effects resulting from increased chromosome copy number may result in altered growth rate [19], transcription or translation [20], virulence [19], and mutation rates [21]. Since chromosome abundance can have far-ranging impacts on bacterial physiology, we hypothesize that it could be one variable that explains, at least in part, the enhanced tolerances of persisters.

To explore the impact of ploidy on bacterial persistence, we describe methods here that can be used to segregate bacteria based on chromosomal content and assess the fidelity of sorted subpopulations. We also describe assays that can be conducted on those subpopulations, along with their respective controls, which can be used to assess whether persistence is influenced by chromosome copy number. We postulate that the majority of these techniques will be broadly applicable to different bacteria and different types of persistence (e.g., β-lactam, fluoroquinolone, aminoglycoside, persistence in growing or starved populations), but also note the current limitations of the methods and areas for improvement.

2 Materials

2.1 Bacterial Strains and Plasmids

Escherichia coli K-12 strain MG1655 is used herein for DNA staining and persistence assays. Other genetically tractable organisms should be amendable to these approaches, although we list caveats where appropriate. To verify chromosomal content microscopically, we describe the use of an origin reporter strain, ori1-*parS*, which has a chromosomally integrated *parS* site located at position 3928826 near the origin of replication in an MG1655 background [22, 23]. When used in combination with pALA2705, which

harbors GFP-ParB under the control of a lac-repressible promoter, distinct fluorescent foci, indicative of the number of chromosomal origins within single cells, can be observed using confocal fluorescent microscopy. Appropriate controls for the origin reporter strain should demonstrate that both GFP-ParB expression and the genomically integrated *parS* site are required for foci formation. Since persistence is measured based on colony forming units (CFUs), we note that any strain selected for chromosomal verification should be capable of being stained with Hoechst 33342 when alive (e.g., confirm that Hoechst 33342 can penetrate the cell membrane to stain DNA for cell sorting, without losing fluorescence over time due to excess efflux pumps) and should be compatible with a functional *par* system (*see* **Note 1**).

2.2 Media

1. LB medium for planktonic growth: 10 g tryptone, 5 g yeast extract and 10 g NaCl in 1 L of MilliQ water. Autoclave for 30 min at 121 °C.
2. 5× M9 minimal salt solution: 33.9 g/L Na_2HPO_4, 15 g/L KH_2PO_4, 5 g/L NH_4Cl, 2.5 g/L NaCl. Autoclave for 30 min at 121 °C.
3. M9 glucose medium for planktonic growth: Combine 1× M9 minimal salts, 0.1 mM $CaCl_2$, 2 mM $MgSO_4$ and 10 mM glucose in a total volume of 400 mL. Filter-sterilize the medium using a 0.22 μm filter upon preparation (*see* **Note 2**).
4. Agar.

2.3 Nucleic Acid Staining

1. 10 mg/mL Hoechst 33342 stock solution (2-′-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1H-benzimidazole trihydrochloride trihydrate; ThermoFisher).
2. PicoGreen.
3. Flow cytometry tubes (Corning Falcon #352054 or similar).

2.4 Persister Assay

1. For retention of plasmid pALA2705, use ampicillin (AMP) at a final concentration of 100 μg/mL in autoclaved MilliQ water. Any antibiotic can be used for persistence assays; as an example, we demonstrate persistence in Fig. 1 using moxifloxacin (MOXI) (*see* **Note 3**). Make antibiotic stocks fresh the day of the experiment and filter-sterilize using a 0.22 μm filter.
2. PBS.
3. Test tubes (glass and/or polypropylene).
4. 250 mL baffled flasks.
5. 96-well round-bottom plates.
6. Microcentrifuge tubes (Eppendorf, 1.5 mL).
7. Syringes.

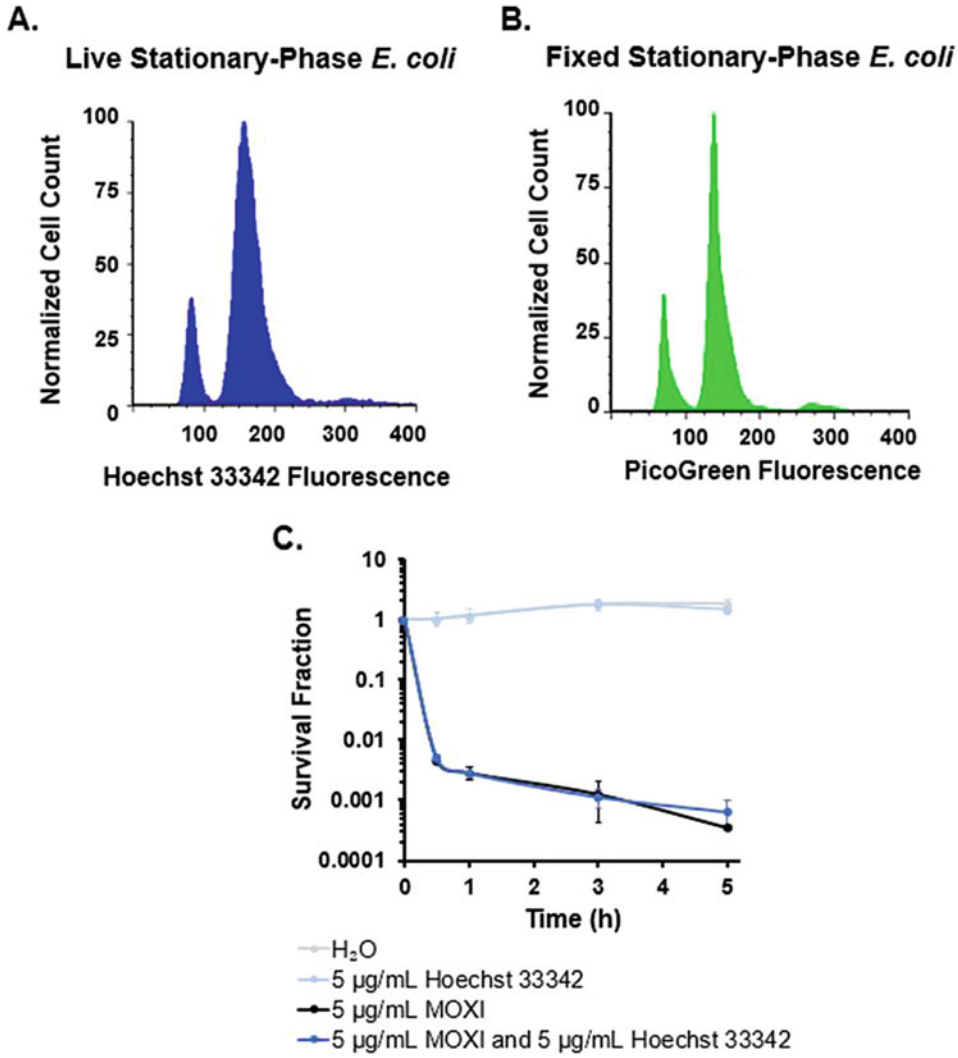

Fig. 1 Selection of Hoechst 33342 as a representative fluorescent dye to quantify chromosome numbers in single cells. (**a**) Representative DNA histogram of live stationary-phase MG1655 grown for 20 h in M9 glucose medium and stained with 5 μg/mL Hoechst 33342. Staining with Hoechst 33342 (**b**) recapitulates the staining pattern observed with a secondary DNA-specific dye, PicoGreen, and (**c**) does not impact culturability or the persistence kill curve when assessed in conjunction with 5 μg/mL MOXI treatment

8. 0.22 μm filter units.
9. 15 mL and 50 mL falcon tubes.
10. Square agar plates.

2.5 Fluorescence Activated Cell Sorting (FACS)

1. 1 μm Yellow-Green fluorescent beads (Polysciences, Inc., Warrington, PA Catalog #17154) or similar.
2. To excite Hoechst 33342, use a 355 nm laser run at 60 mW power (Coherent, Santa Clara, CA) and collect fluorescence

intensities using a 410 nm long pass filter and a 450/50 nm bandpass filter.

3. To excite PicoGreen, use a 488 nm laser run at 100 mW power (Coherent, Santa Clara, CA) and collect fluorescence intensities using a 505 nm long pass filter and a 525/50 band pass filter.
4. 70% ethanol.
5. 4% paraformaldehyde (PFA) in PBS.

2.6 Microscopy

1. Plain microscopy slides.
2. Cover glass.
3. Clear nail polish.

3 Methods

3.1 Cell Growth Conditions

We chose to work with nongrowing bacterial populations for chromosome counting and segregation, because cells in such populations have a unit number of chromosomes, in contrast to growing populations, which have both fully and partially replicated chromosomal copies [9, 10].

1. Inoculate the bacterial strain from a 25% glycerol stock (−80 °C) into 2 mL of LB in a test tube. Incubate at 37 °C for 4–5 h with shaking (250 rpm).
2. Measure the OD_{600} of cells and calculate the volume of cells required to inoculate 25 mL of medium to an OD_{600} ~ 0.01.
3. Aliquot the calculated volume into a microcentrifuge tube and spin at 15,000 rpm for 3 min.
4. Remove the supernatant.
5. Resuspend the bacterial pellet in 300 μL of medium, and inoculate into 25 mL of M9 glucose medium in a 250 mL baffled flask. Incubate at 37 °C with shaking (250 rpm) for 20 h (*see* **Note 4**).
6. After 20 h of growth, remove 500 μL of cells and spin in a microcentrifuge tube at 15,000 rpm for 3 min.
7. Remove the supernatant.
8. Resuspend the bacterial pellet in 500 μL of PBS.
9. To determine the final cell density, use 10 μL of cell culture and measure the OD_{600} (*see* **Note 5**).
10. To assess culture viability, perform serial five- or tenfold dilutions in PBS to determine CFUs/mL. As an example of using fivefold dilutions, in the first well of a 96-well round-bottom plate, dilute 20 μL of cells in 80 μL of PBS, and mix well. Then

using new pipette tips, transfer 20 μL of the diluted cells into 80 μL of PBS in the second well, and mix well. Repeat for nine wells. Plate 10 μL of each dilution onto LB agar for CFU enumeration. Incubate at 37 °C for 16–24 h (*see* **Notes 6** and **7**). During stationary phase, we expect ~10^9 CFUs/mL.

3.2 Nucleic Acid Staining Selection

Here we describe the use of Hoechst 33342 to quantify chromosomal content in living, individual *E. coli* cells. Selection of Hoechst 33342 was based on its low toxicity, cell membrane permeability, lack of impact on the persistence phenotype we were interested in studying, and its ability to recapitulate the staining pattern of PicoGreen, which is a benchmark double stranded (ds) DNA-specific dye that is often used to quantify chromosomes in fixed cells [4, 24–27] (Fig. 1). Hoechst 33342 is a cell-permeable stain that binds to AT-rich regions of the minor groove of DNA, is excited by ultraviolet (UV) light, and emits blue fluorescence within the 460 nm to 490 nm range [28, 29]. It is often used for live-cell microscopy [30–32] and flow cytometric cell cycle analysis [33–35] in mammalian cells. PicoGreen is an alternative dsDNA-specific dye with an excitation of 480 nm and an emission spectrum at 520 nm, but it is not a good candidate for live-cell analysis because it has difficulty permeating intact cell membranes [36]. Other fluorescent stains, including TOTO-1, YOYO-1, and dyes of the Syto series, fluoresce upon binding to nucleic acids, yet may have difficulty discriminating between DNA and RNA [37–42]. Importantly, dsDNA-specific fluorescent stains other than Hoechst 33342 or alternative fluorescent methods to assess ploidy may be developed or work with other bacterial strains and/or conditions (*see* **Note 8**). However, it is important to stress that any stain or fluorescent probe used should not alter untreated CFU/mL levels under the conditions of interest, should not alter antibiotic kill curves that are used for persister enumeration under the conditions of interest, and should recapitulate the chromosomal histograms of a benchmark dsDNA stain, such as PicoGreen, under the conditions of interest.

3.2.1 Hoechst 33342 Staining Procedure

1. Grow bacteria to stationary phase as described in Subheading 3.1.
2. Dilute Hoechst 33342 1:10 in autoclaved MilliQ water and add to the culture to a final concentration of 5 μg/mL. For unstained controls, add an equal volume water. Stain in the dark for at least 30 min at 37 °C.

3.2.2 Determination of the Impact of Hoechst 33342 Staining on Culturability and Persistence

The hallmark of bacterial persistence is the presence of biphasic killing kinetics. When studying the impact of nucleic acid stains on persistence, it is important to determine whether the stain alters the culturability of untreated cells or the survival curve of treated cells (Fig. 1). Sampling multiple time points throughout the course

of the experiment will allow for the determination of an impact on persistence, whereas monitoring the culturability of an untreated control stained with Hoechst 33342 will indicate any toxicity. In the conditions considered here, we assessed the impact of Hoechst 33342 on stationary-phase culturability and persistence toward MOXI. If other growth conditions, strains, and/or antibiotics are desired, comparable controls should be conducted.

1. Grow bacteria to stationary phase as described in Subheading 3.1, and prepare the following 25 mL cultures:
   (a) Untreated and Unstained.
   (b) Untreated and Stained with 5 μg/mL of Hoechst 33342.
   (c) Treated with 5 μg/mL of MOXI and Unstained.
   (d) Treated with 5 μg/mL of MOXI and Stained with 5 μg/mL of Hoechst 33342.
2. Prior to staining and/or treatment, remove 500 μL samples from each culture, add to microcentrifuge tubes, and centrifuge at 15,000 rpm for 3 min.
3. Measure the OD_{600} and determine initial CFUs as described in **steps 7–10** of Subheading 3.1.
4. Add Hoechst 33342 to a final concentration of 5 μg/mL or an equal volume of water.
5. Immediately treat cultures with a final concentration of 5 μg/mL MOXI or equal volume of water (*see* **Note 9**).
6. Incubate cultures in the dark at 37 °C with shaking (250 rpm) for 5 h (*see* **Note 10**).

At various time points during the persistence assay:

7. Remove 500 μL of culture and spin at 15,000 rpm for 3 min in a microcentrifuge tube.
8. Remove the supernatant.
9. Resuspend the pellet in 500 μL of PBS.
10. Spin again at 15,000 rpm for 3 min.
11. Repeat **steps 8–10** until the residual antibiotic concentration is below the minimum inhibitory concentration (MIC) (*see* **Note 11**).
12. Perform serial dilutions as described in **step 10** of Subheading 3.1.
13. Incubate LB agar plates with spotted dilutions at 37 °C for 16–24 h.
14. Enumerate CFUs after 16–24 h.
15. Calculate survival fractions by dividing the number of CFUs at the final time point by the number of initial CFUs.

16. Compare survival curves to determine whether staining impacts culturability.

In addition to assessing the impact of staining on persistence, these survival curves can be used to provide an estimate of the number of cells that would need to be sorted during FACS to ensure the number of persisters observed is above the limit of detection (*see* Subheading 3.7).

3.3 Sample Preparation for Fluorescence Activated Cell Sorting (FACS)

A diagrammatic overview of the cell sorting procedure is outlined in Fig. 2.

3.3.1 Cell Dilution and Staining for FACS

1. Grow bacteria to stationary phase as described in Subheading 3.1.
2. Pipet 500 μL of samples of the stationary-phase culture into microcentrifuge tubes, and centrifuge at 15,000 rpm for 3 min.
3. Measure the OD_{600}. Calculate the volume of culture required to inoculate 5 mL of PBS to an $OD_{600} < 0.03$ in a 15 mL falcon tube (*see* **Note 12**). Prepare two falcon tubes of cells for stained and unstained samples.
4. Stain the cells with 5 μg/mL of Hoechst 33342 (which has been diluted 1:10 in water) or add an equal volume of water for unstained cells.
5. Incubate stained and unstained cells in the dark at 37 °C for 30 min.
6. Transfer up to 4 mL of stained and unstained cells into flow cytometry tubes for FACS.
7. Transport samples to the cell sorter in a dark container heated to ~37 °C (*see* **Note 13**).

3.3.2 Other Preparations for FACS

Cells are sorted into the collection tubes in droplets of PBS sheath fluid, which for the stationary-phase cultures discussed here, should be collected into an equal volume of sterile spent medium for the subsequent assays. It is best practice to determine what approximate volume a given number of cells will yield from the sorter. For the sorting conducted here, 1,000,000 sorted cells resulted in ~1 mL of PBS, and therefore, flow cytometry tubes containing 1 mL of sterile spent medium were prepared for cell collection.

1. While cells are staining (Subheading 3.3.1), spin the remaining culture (~20 mL due to overnight evaporation) in a 50 mL falcon tube at 4000 rpm for 10 min at room temperature.

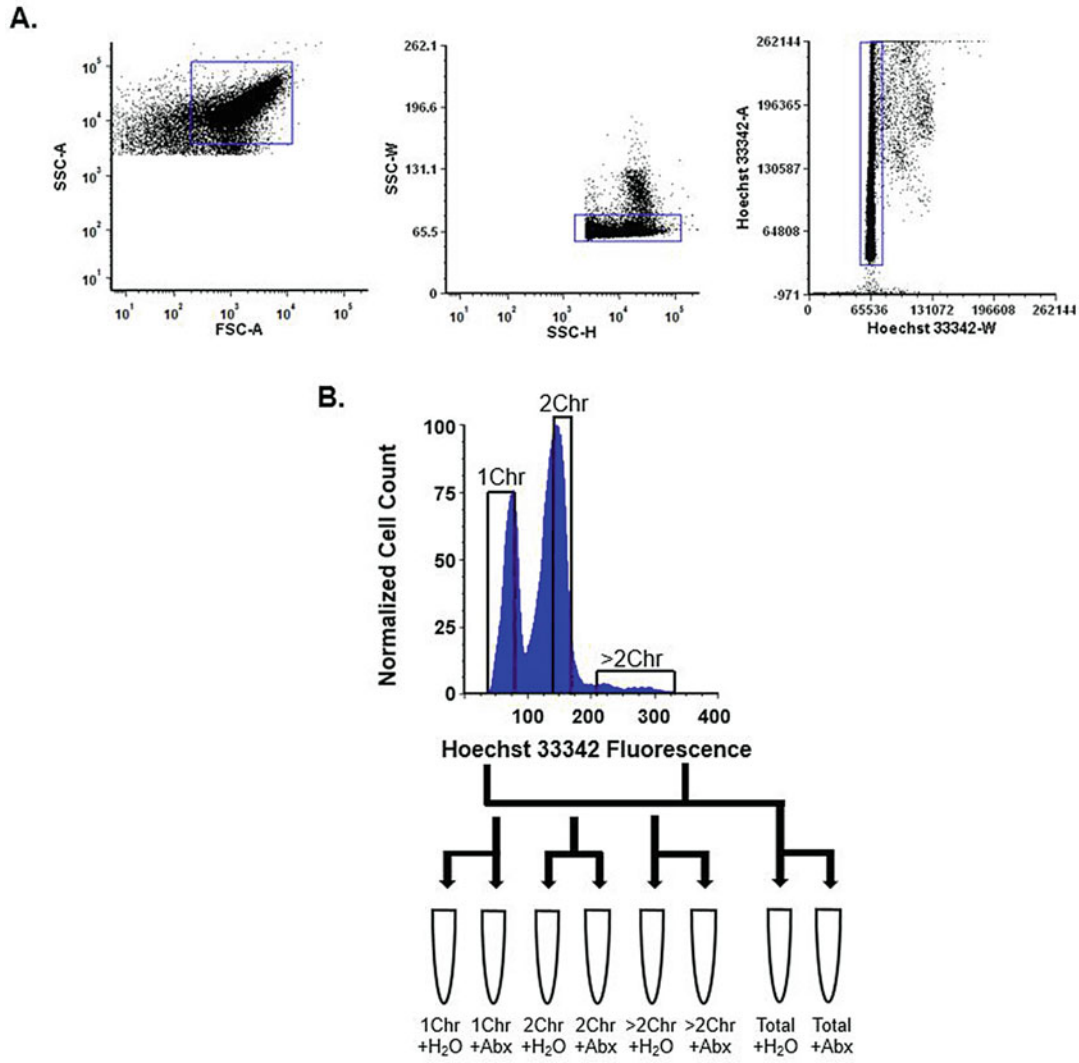

Fig. 2 Method to sort stationary-phase *E. coli* based on chromosomal content. (**a**) Suggested gating strategy to sort cells based on chromosomal content as determined by staining with 5 μg/mL Hoechst 33342. First, identify cells using FSC-A and SSC-A gating, and assign this gate to the next plot. To minimize cell aggregates from subsequent plots, gate next using SSC-H and SSC-W, followed by nested gating with Hoechst 33342 as determined by Hoechst 33342-W and Hoechst 33342-A. (**b**) Display this gated single-cell Hoechst 33342 stained population on a Hoechst 33342-A versus count histogram, and draw 1Chr, 2Chr, and >2Chr gates for sorting the populations of interest. A representative gating strategy to minimize gate leakage is portrayed. Collect samples in flow cytometry tubes containing sterile spent medium, and divide each sorted population into treated and untreated subpopulations for your assay. 1Chr, 2Chr, and >2Chr indicate chromosome number (Chr). *Abx* antibiotic

2. Filter-sterilize the supernatant (spent medium) using an 0.22 μm filter into a 50 mL falcon tube.
3. Add 1 mL of sterile spent medium to labeled flow cytometry collection tubes, and bring these tubes to the sorter for cell

collection. These tubes will be used to collect the sorted cells, such that the final volume in these tubes after sorting 1,000,000 cells will be 2 mL of 50% PBS/50% spent medium (*see* **Note 14**).

3.4 Controls for FACS: Determination of Whether the Timing Required to Sort Cells Using FACS Influences Persistence

Depending on the strain, the number of cells sorted, and the flow rate, the cell sorting process can take several hours or longer. For the conditions and sorter we used, sorting 1,000,000 wild-type cells took up to an hour in four-way purity sort mode. To determine whether this timing impacts the persistence phenotype of interest, we recommend conducting "presort" and "postsort" persister assay controls on unsorted cells. Briefly, dilute stained and unstained cells in 50% PBS/50% sterile spent medium to an OD_{600} that approximates the OD_{600} of the cells that were isolated from the sorter and collected in equal volume sterile spent medium. Subsequently, perform persister assays on these samples (*see* Subheading 3.7). "Presort" indicates that a persistence assay is initiated immediately prior to cell sorting, and "postsort" indicates that a persistence assay is initiated immediately after the conclusion of cell sorting. Both presort and postsort persistence assays should be performed on stained and unstained cells that are not sorted, and they are initiated at different points in time. The persister fractions of the presort condition can then be compared to those of the postsort condition to determine whether the timing required for cell sorting impacts persistence. These data can also be compared to the persister fraction arising from a Total Sort population to verify whether sorting itself influences persistence. *See* Subheading 3.7 for more details.

3.5 FACS

FACS enables the segregation of bacteria into smaller subpopulations based on a phenotypic characteristic that is quantitatively measured via a fluorescence value. Follow your manufacturer's guidelines for proper sorter start-up, alignment/QC, and sort setup. For each FACS experiment, take special consideration of the biosafety level of the organism you work with; FACS functions under a pressured system which can produce aerosols during a sort failure, such as a nozzle clog. Accidental inhalation of these droplets can cause unintentional risks to your health. A proper risk assessment should be performed with your institutional biosafety officer prior to conducting sort experiments and all safety recommendations should be implemented. Additionally, proper precautions should be adhered to in order to ensure that the FACS sorter remains sterile and free from particulate matter (*see* **Note 15**). Prior to and after each sort experiment, the sorter should be cleaned with a freshly prepared 10% bleach solution for 10 min, followed by a 10 min wash with sterile PBS or autoclaved DI water. Weekly sterilizations of the sheath fluid container via autoclave and weekly sterilization of the fluid lines with 70% ethanol are

recommended, as per your manufacturer's instructions. Frequent incubation of sheath fluid captured into a tube from above the waste catcher on an LB agar plate at 37 °C for 16–24 h is recommended. A lack of colony growth confirms a sterile sorter. Regular maintenance on the sorter, including laser alignment, with a particular focus on the UV laser if Hoechst 33342 is to be utilized, is recommended.

1. Turn on the FACS sorter ~30 min early to allow the sorter and UV laser to warm up, according to the manufacturer's instructions (*see* **Note 16**).
2. Set the laser delay values, area scaling factors, droplet breakoff position, and drop delay values according to the instrument's instructions.
3. Obtain single-cell Hoechst 33342 fluorescent values by exciting each cell with a 355 nm laser run at 60 mW power (Coherent, Santa Clara, CA) and filtering emitted light through a 410 nm long pass filter and a 450/50 nm bandpass filter.
4. If your sorter has temperature control options, select a temperature at which you wish to sort the bacteria (*see* **Note 17**).
5. Select the appropriate sorter nozzle and pressure for sorting bacteria (*see* **Note 18**).
6. To place area measurements on the same scale as height measurements for each sorting session, optimize forward scatter (FSC) and UV area scaling factors using 1 μm Yellow-Green fluorescent beads (*see* **Note 19**).
7. Set FSC and side scatter (SSC) parameters to be recorded on a log scale. Adjust Hoechst 33342 fluorescence values to be recorded on a linear scale (*see* **Note 20**).

   To identify cells:

8. Run a sample of clean, filtered PBS to assess particulate contamination in tubing and electronic noise. Adjust FSC and SSC voltages and threshold values to minimize electronic noise.
9. Identify bacterial cells using FSC and SSC as determined by an unstained control (*see* **Note 21**) and adjust voltages to visualize the entire population.
10. Exclude cell aggregates by gating first on SSC-H and SSC-W, followed by gating with Hoechst 33342 as determined by Hoechst 33342-W and Hoechst 33342-A (*see* **Note 22**).
11. Place a sample of unstained, live stationary-phase *E. coli* on the sorter and adjust voltages such that electronic noise is minimized and cells are on scale (*see* **Note 23**). Record a data file of at least 50,000 cells.

12. Backflush the system sample line to ensure unstained cells are removed from the tubing.
13. Place the stained sample on the sorter and adjust both doublet discrimination gates if necessary. Set gates representative of the one-chromosome population and the two-chromosome population, as demonstrated in Fig. 2. Employ a conservative gating strategy to ensure as minimal leakage between the gates as possible.
14. Sort 1,000,000 cells per gate (*see* Subheading 3.7), and collect cells in 1 mL of sterile spent medium in a flow cytometry tube (these tubes were prepared in Subheading 3.3.2). After sorting, place the cells back in the dark container.
15. For a Total Sort sample, sort 1,000,000 cells as determined by the SSC-H and SSC-W and the Hoechst 33342-W and Hoechst 33342-A gates. Collect cells in 1 mL of sterile spent medium in a flow cytometry tube.

3.6 Verification of Sorting Fidelity Based on DNA Content

To assess the fidelity of the sorting methodology, we recommend using two methods to evaluate sorting precision. First, we suggest restaining sorted cells with a secondary dsDNA-specific dye and analyzing the restained subpopulations using flow cytometry. In our experiments, we restained the sorted Hoechst-stained subpopulations with PicoGreen, a known cell-impermeable dsDNA-specific dye (*see* **Note 24**). Second, to quantify sort purity using a method independent of staining, we recommend sorting a strain where chromosome copy number is reported by a fluorescent protein. In our experiments, we used an origin reporter strain that has distinct fluorescent foci indicative of the number of chromosomes within single cells, and visualized the subpopulations using confocal fluorescence microscopy. Both methods are described herein.

3.6.1 Confirmation of DNA Content of Sorted Cells Using a Secondary dsDNA-Specific Dye (PicoGreen)

1. Grow bacteria to stationary phase as described in Subheading 3.1.
2. After 20 h, remove 1 mL, spin for 3 min at 15,000 rpm in a microcentrifuge tube, and fix in 1 mL of 4% PFA for 30 min at room temperature (*see* **Note 25**).
3. Centrifuge the cells for 3 min at 15,000 rpm.
4. Resuspend in 1 mL of PBS.
5. Stain the cells with 5 μg/mL of Hoechst 33342 and prepare for FACS, as described in Subheading 3.3.
6. Sort 1,000,000 cells per gate based on Hoechst 33342 fluorescence as described in Subheading 3.5. Collect in empty flow cytometry tubes.

7. Permeabilize the sorted cells with 9 mL of 70% ethanol in a 15 mL polypropylene conical tube for at least 3 h at 4 °C (*see* **Note 26**).
8. Spin the permeabilized cells at 4000 rpm for 10 min at 4 °C.
9. Remove 9 mL of supernatant.
10. Resuspend the pellet and transfer the remaining ~1 mL of cells to a microcentrifuge tube.
11. Spin for 3 min at 15,000 rpm.
12. Remove the supernatant.
13. Leave the tubes open overnight and cover with low lint tissue to allow residual ethanol to evaporate.
14. The next morning, resuspend the dried pellet in 500 μL of PBS.
15. Dilute a PicoGreen dimethyl sulfoxide (DMSO) stock 1:100 in 25%/75% DMSO/MilliQ water. Stain the ethanol-fixed cells in PBS with 100 μL of the diluted PicoGreen solution or 100 μL of solvent for an unstained control. Incubate in the dark at room temperature for ~3 h.
16. Centrifuge the cells for 3 min at 15,000 rpm.
17. Remove 500 μL of supernatant.
18. Resuspend the cell pellet in the remaining 100 μL and transfer to a de-capped PCR tube placed into a flow cytometry tube.
19. Acquire single-cell flow cytometric data:
    (a) Turn on the cytometer ~30 min early to allow the cytometer and lasers to warm up and perform a QC check as per manufacturer's instructions (*see* **Note 27**).
    (b) Identify single cells using FSC and SSC and perform doublet discrimination as described in Subheading 3.5. Set the PicoGreen channel to linear scaling.
    (c) Apply no gates to the sort-check samples (*see* **Note 28**).
    (d) Acquire single-cell PicoGreen fluorescence values by exciting each cell with a 488 nm laser run at 100 mW power (Coherent, Santa Clara, CA) and collect using a 505 nm long pass filter and a 525/50 nm bandpass filter. Evaluate fidelity of sorted populations.

3.6.2 Confirmation of DNA Content of Sorted Cells Using an Origin-of-Replication Reporter

1. Grow origin reporter strain ori1-*parS* + pALA2705 in M9 minimal glucose medium in the presence of 100 μg/mL of AMP for plasmid retention as described in Subheading 3.1 (*see* **Note 29**).
2. Fix the cells with 4% PFA as described in **steps 2–4** of Subheading 3.6.1.

3. Stain with 5 μg/mL of Hoechst 33342 as described in Subheading 3.3.1.
4. Using FACS (Subheading 3.5), sort 2,000,000 of one-chromosome, two-chromosome, and Total Sort subpopulations each into empty flow cytometry tubes (*see* **Note 30**).
5. Centrifuge the eluted cells for 3 min at 15,000 rpm and remove all but ~30 μL of supernatant. Store in the dark at 4 °C until microscopic analysis.
6. On the day of microscopic analysis, dissolve 0.1 g of agarose in 10 mL of autoclaved MilliQ water by microwaving for ~1 min in a 50 mL falcon tube.
7. After cooling slightly, pipet 1 mL of agarose solution onto sterile plain microscopy slides.
8. Lay a second plain microscopy slide cross-wise over top by supporting with adjacent double-stacked plain microscopy slides laid parallel to and on both sides of the original slide.
9. Allow to harden at room temperature for ~1 h.
10. Remove the top slide carefully, and cut the agarose pad into a ~2 × 2 cm^2.
11. Pipet 2 μL of the fixed, sorted, and concentrated ori1-*parS* + pALA2705 cell suspensions onto the agarose pad and allow to air dry.
12. Pipet another 2 μL of cell suspension on top of the original spot to increase cell density, allow to air dry. Repeat a third time.
13. Overlay a cover glass and seal with clear nail polish.

3.6.3 Controls for Origin Reporter

1. Grow MG1655 with pALA2705 (no *parS* binding site) and ori1-*parS* with pALA2705Δ*parB* (contains *parS* binding site and expresses GFP but lacks ParB) to stationary phase as described in Subheading 3.1. Use 100 μg/mL of AMP for plasmid retention.
2. Fix in 4% PFA as described in **steps 2–4** of Subheading 3.6.1 and resuspend in PBS for microscopic analysis.
3. Dilute these samples 1:12 in PBS.
4. Prepare agarose microscopy slides as described in **steps 6–10** in Subheading 3.6.2.
5. Spot 2 μL onto the agarose and allow to air dry.
6. Overlay with a cover glass and seal with clear nail polish.

3.6.4 Confocal Microscopy

1. Visualize the origin reporter strain using confocal fluorescence microscopy (*see* **Note 31**). We recommend observing the bacteria using phase contrast and visualizing the foci by taking a Z-stack using the 488 nm laser (*see* **Notes 32–34**). A

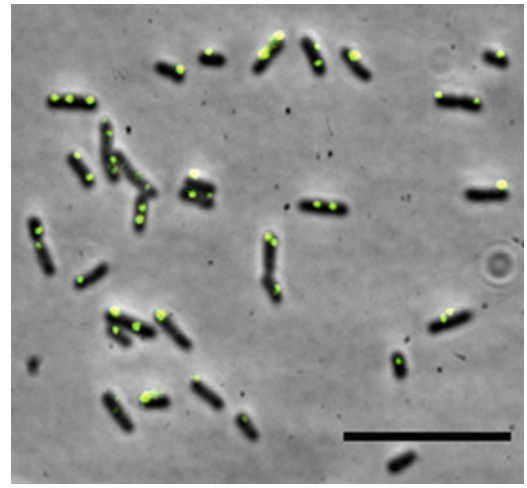

Fig. 3 Representative phase contrast image with fluorescent overlay of origin reporter strain ori1-*parS* with the *parS* site located at position 3928826. Scale bar represents 5 μm

representative confocal fluorescence microscopy image of an unsorted, stationary-phase origin-reporter strain is portrayed in Fig. 3.

2. Use image analysis software, such as MicrobeJ, to count foci within single cells (*see* **Note 35**), and correlate this data with the PicoGreen sort check data (Subheading 3.6.1).

3.7 Culturability and Persistence Assays on Cell Samples

In this section, you will want to use persister fractions measured from the assays conducted in Subheading 3.2.2 to estimate the number of persisters you would expect to observe from the number of cells you choose to sort. You can then calculate the estimated number of persisters you expect to count at each dilution when plating serial dilutions, keeping in mind the number and volume of cells in the assay, whether the sample is divided into treated and untreated conditions, and the volume of cells plated for CFU measurements. If taking hourly time points where survival is higher during earlier time points, you may also want to plate additional dilutions. If your persister fraction is very low, we recommend only taking initial and final CFUs (no intermediate time points), and plating all remaining volume at the final time point. Controls with cells that were not sorted but treated under identical conditions as sorted cells can assess whether biphasic survival curves are attained under those treatment conditions.

3.7.1 Culturability and Persistence Assay on Sorted Fractions

Once the cells have been sorted based on chromosomal content, proceed with assays that address your question of interest. As an example, we provide instructions on how to perform persistence assays on the sorted subpopulations.

1. Divide each sorted sample from Subheading 3.5 (which, in our case, is 2 mL in total volume) into two 1 mL aliquots in 15 mL polystyrene tubes to represent treatment and control conditions.
2. Remove 100 μL of cells from each tube and add to microcentrifuge tubes (initial samples for initial CFU/mL quantifications).
3. Treat each culture (**step 1**) with your antibiotic of choice or an equal volume control solvent.
4. Incubate cultures at 37 °C with shaking at 250 rpm for 5 h.
5. Process initial samples by adding 900 μL of PBS to the microcentrifuge tubes, centrifuging at 15,000 rpm for 3 min, and removing 900 μL of supernatant. Resuspend the pellet in the remaining 100 μL of PBS, and perform serial dilutions as described in **step 10** of Subheading 3.1. Spot 10 μL of each dilution for the first six wells onto LB agar, and incubate the agar plates for 16–24 h at 37 °C.
6. At various time points during the assay, remove 100 μL of cell culture from each tube and add to microcentrifuge tubes.
7. Add 900 μL of PBS.
8. Spin at 15,000 rpm for 3 min.
9. Remove 900 μL of supernatant.
10. Repeat **steps 7–9** until the residual antibiotic concentration in the treated cultures is below the MIC (*see* **Note 11**) and perform the same number of washes on the untreated controls.
11. Resuspend the pellet in the remaining 100 μL. For the untreated samples, perform serial dilutions and plate 10 μL of each dilution onto LB agar and allow to dry. For the treated samples, process identically and plate 50 μL of each dilution, instead of 10 μL to increase the limit of detection (*see* **Note 36**).
12. Incubate the agar plates for 16–24 h at 37 °C, and count CFUs at the appropriate dilution (between 20–100 CFUs) the following day.

3.7.2 Culturability and Persistence Assays on Unsorted Cells Diluted to the Same Density of Sorted Cells

These steps can be conducted in parallel with the sorted cell persister assays described in Subheading 3.7.1.

1. To demonstrate that FACS sorting does not alter the persistence phenotype, dilute the stained and unstained cells samples that had been FACS analyzed and/or sorted to an $OD_{600} < 0.001$ in 2 mL of 50%/50% PBS/sterile spent medium.
2. Follow **steps 1–12** in Subheading 3.7.1.

4 Notes

1. Other fluorescent dyes and fluorescent proteins that form foci can be used to quantify chromosomal content. However, one should ensure that the dyes are nontoxic and that foci are produced within the vast majority of cells.
2. Any medium can be used, but we note that the rate of growth within the culture may affect chromosome abundances.
3. We note that any antibiotic can be used for persistence assays, and recommend that the antibiotic concentration be high enough such that persister quantification occurs in the concentration-independent portion of survival plots (regime where modest changes to antibiotic concentration do not alter survival appreciably). For the persistence assay portrayed in Fig. 1, a 5 mg/mL stock of MOXI was made by dissolving it in autoclaved MilliQ water until fully dissolved, and samples were treated at a final concentration of 5 μg/mL. Further, biphasic killing kinetics must be observed for treatments to yield persister measurements.
4. We recommend using medium that was freshly prepared less than a week beforehand. In addition, the length of time to reach stationary phase varies with different strains and different bacteria; 20 h is a suggestion and works well for *E. coli* K12 MG1655, but can be shortened or extended as necessary.
5. We generally dilute 10 μL of cells into 290 μL of PBS in a flat-bottom 96-well plate before measuring the OD_{600} using a spectrophotometer. We calculate the OD_{600} by accounting for the 30-fold dilution.
6. For CFU enumeration, we recommend drying LB agar plates covered and at room temperature for ~2 days prior to use in order to prevent 10 μL spots from running together.
7. We generally count dilutions that contain 20–100 bacteria.
8. Fluorescent DNA binding proteins, such as HU-GFP, have also been previously used to assess DNA content within single cells [43, 44].
9. We observed that the age and method of storage for antibiotic solutions can affect killing kinetics, especially for persistence assays. We therefore recommend making antibiotic stocks fresh on the day of the experiment.
10. Additional time points beyond 5 h of treatment may be required to see biphasic killing kinetics.
11. Calculate the MIC for each strain tested using agar or broth dilution methods [45], or an antibiotic-specific Etest strip (bioMérieux, Marcy-l'Étoile, France).

12. Check the sorter manufacturer's guidelines for appropriate cell density for cell sorting. We determined that 2.5×10^7 to 3×10^7 cells/mL is a good density for cell sorting with the machine used here (*see* **Note 16**).
13. We used prewarmed heating packs placed in Styrofoam containers to transport cells at desired temperatures.
14. Provide extra empty flow cytometry tubes and flow cytometry tubes containing filter-sterilized spent medium as backup.
15. To assess the sorter's sterility prior to sorting, when arriving at the sorter obtain a sample of sheath fluid directly from the sheath fluid stream by catching several mL into a clean tube and plating directly onto LB agar. Sterility should be observed after incubation at 37 °C for 16–24 h.
16. We performed sorting with a BD Biosciences FACSAria Fusion Special Order Research Product (SORP) (San Jose, CA) system with a 70 μm nozzle at 70 psi and window extension 10.0. We used FACSDiva version 8.0 software to acquire and analyze the data. Since each FACS sorter is unique, we strongly recommend testing and optimizing specific settings prior to conducting all sort experiments.
17. Per our sorter's capabilities, we kept our sort sample and collection tubes at 37 °C.
18. We used a 70 μm nozzle and sorted bacteria at 70 psi system pressure using the FACSAria Fusion.
19. Adjust the area scaling factors for the height (H) and area (A) signals for each detector until the median signal intensity values of the corresponding H and A signals are equivalent in order to place area measurements on the same scale as height measurements.
20. Forward scatter (FSC) is relatively proportional to a particle's cross-sectional area and refractive index, whereas side scatter (SSC) is relatively proportional to a particle's internal complexity or granularity. Small particles such as bacteria are best visualized with FSC and SSC parameters set to a log scale. We used a linear scale to observe DNA content via Hoechst 33342 staining to evenly space fluorescence peaks along the *x*-axis of a histogram, which correlate with integer numbers of chromosomes.
21. With our conditions, we set signal thresholds to SSC 200 and DAPI 1000 channels for Hoechst 33342–stained cells and to SSC 200 and DAPI 200 channels for unstained cells. The threshold values need to be optimized on all instruments to ignore electronic noise and record data for all bacterial cells.
22. For DNA content analysis where we want to observe a twofold change in DNA content, it is important to create doublet

discrimination plots and reduce the likelihood of sorting cells that are clumped together. For example, we want to ensure that a diploid signal we observe is due to the presence of a cell harboring two chromosomes, and not because two monoploid cells are stuck together. To do so, we recommend gating on SSC-H and SSC-W, followed by gating with Hoechst 33342 as determined by Hoechst 33342-W and Hoechst 33342-A (Fig. 2a).

23. Monitoring the Threshold Rate (events per second) gives an indication of the amount of electronic noise present based on the parameter threshold and voltages selected. As FSC and SSC voltages are adjusted, the events/second rate may greatly increase, indicating that electronic noise is surpassing the threshold value(s).
24. Both dsDNA-specific dyes should have distinct fluorescent properties (e.g., unique excitation and emission spectra). For concomitant use, the dye and the fluorescent protein should also be fluorometrically distinct.
25. For verification of sorting fidelity, fixation is not required and live cell populations can instead be used. We fixed the cells for convenience, so as to not have to rush to permeabilize the cells for staining with PicoGreen.
26. While the cells are already fixed in PFA before Hoechst 33342 staining, PicoGreen requires permeabilization with ethanol to access and bind to the intracellular DNA. Both fixation methods are required when using these fluorescent dyes together.
27. We used an LSRII SORP (Special Order Research Product) flow cytometer and Cytometer Setup and Tracking Beads for QC (BD Biosciences, San Jose, CA).
28. Since we are conducting a purity check, no gates should be applied to the reanalyzed samples. However, doublet discrimination gates may be necessary if cells have clumped during preparation steps between sorting and flow cytometry.
29. For strain ori1-*parS* + pALA2705, induction with IPTG is not required to observe foci, as previously determined [22].
30. We recommend increasing the number of cells sorted when subsequently visualizing these cells using confocal microscopy in order to increase cell density and ensure multiple cells can be visualized in each frame.
31. We used a Nikon Ti-E (Nikon Instruments, Melville, NY) equipped with a Yokogawa spinning disc (CSU-21) (Yokogawa, Tokyo, Japan) mounted with a quad dichroic beam splitter accommodating 405, 488, 561, and 647 lasers (Chroma, Bellows Falls, VT).
32. In our setup, the microscope is controlled using NIS-Elements, V4.5 (Nikon Instruments). The spinning disc

detector is a Hamamatsu ImagEM back-thinned EMCCD (Hamamatsu, Tokyo, Japan). We use an emission filter, ET525/50m (Chroma) for emission discretion after the spinning disc. We use an Agilent laser launch with a 488 nm laser for fluorescence imaging and a piezo stage with 100 μm range capable up to 100 steps/s. We use a CF160 Plan Fluor Phase Contrast DLL 100× oil 1.3NA objective with corresponding annulus.

33. For our purposes, exposure for phase contrast images is set to 300 ms, and 1 s exposure at 50% laser power is used for fluorescence imaging. Exposure and laser power are optimized to limit photobleaching and maximize the signal to noise ratio.
34. Since the origin-of-replication foci within the cells may not be within the same plane, we recommend taking GFP Z-stacks every 0.3 μm for 21 steps.
35. We note that while the origin reporter is a useful tool to quantify chromosomes in untreated and fixed bacteria, certain conditions may cause destruction of the foci, leading to diffuse fluorescence within the cells. If intending to quantify chromosomes, we recommend testing any experimental conditions on the origin reporter strain first to see if the treatment alters foci formation and chromosome abundance enumeration.
36. The plating volume can be adjusted in order to observe 20–100 CFUs at your expected dilution.

Acknowledgments

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References

1. Balaban NQ, Helaine S, Lewis K et al (2019) Definitions and guidelines for research on antibiotic persistence. Nat Rev Microbiol 17:441–448
2. Balaban NQ, Merrin J, Chait R et al (2004) Bacterial persistence as a phenotypic switch. Science 305:1622–1625
3. Keren I, Kaldalu N, Spoering A et al (2004) Persister cells and tolerance to antimicrobials. FEMS Microbiol Lett 230:13–18
4. Barrett TC, Mok WWK, Murawski AM, Brynildsen MP (2019) Enhanced antibiotic resistance development from fluoroquinolone persisters after a single exposure to antibiotic. Nat Commun 10:1177
5. Fisher RA, Gollan B, Helaine S (2017) Persistent bacterial infections and persister cells. Nat Rev Microbiol 15:453–464
6. Wilmaerts D, Windels EM, Verstraeten N, Michiels J (2019) General mechanisms leading

to persister formation and awakening. Trends Genet 35:401–411
7. Helaine S, Kugelberg E (2014) Bacterial persisters: formation, eradication, and experimental systems. Trends Microbiol 22:417–424
8. Amato SM, Fazen CH, Henry TC et al (2014) The role of metabolism in bacterial persistence. Front Microbiol 5:70
9. Mok WWK, Brynildsen MP (2018) Timing of DNA damage responses impacts persistence to fluoroquinolones. Proc Natl Acad Sci U S A 115:E6301–E6309
10. Akerlund T, Nordström K, Bernander R (1995) Analysis of cell size and DNA content in exponentially growing and stationary-phase batch cultures of *Escherichia coli*. J Bacteriol 177:6791–6797
11. Hill NS, Kadoya R, Chattoraj DK, Levin PA (2012) Cell size and the initiation of DNA replication in bacteria. PLoS Genet 8: e1002549
12. Kraemer J, Sanderlin A, Laub M (2019) The stringent response inhibits DNA replication initiation in *E. coli* by modulating supercoiling of *oriC*. mBio 10:e01330
13. Soppa J (2017) Polyploidy and community structure. Nat Microbiol 2:1–2
14. Soppa J (2015) Polyploidy in archaea and bacteria: about desiccation resistance, giant cell size, long-term survival, enforcement by a eukaryotic host and additional aspects. J Mol Microbiol Biotechnol 172730(24):409–419
15. Michelsen O, Hansen FG, Albrechtsen B, Jensen PR (2010) The MG1363 and IL1403 laboratory strains of *Lactococcus lactis* and several dairy strains are diploid. J Bacteriol 192:1058–1065
16. Krisko A, Radman M (2013) Biology of extreme radiation resistance: the way of *Deinococcus radiodurans*. Cold Spring Harb Perspect Biol 5:a012765
17. Ohtani N, Tomita M, Itaya M (2010) An extreme thermophile, *Thermus thermophilus*, is a polyploid bacterium. J Bacteriol 192:5499–5505
18. Krasin F, Hutchinson F (1977) Repair of DNA double-strand breaks in *Escherichia coli*, which requires *recA* function and the presence of a duplicate genome. J Mol Biol 116:81–98
19. Soler-Bistué A, Mondotte JA, Bland MJ et al (2015) Genomic location of the major ribosomal protein gene locus determines *Vibrio cholerae* global growth and infectivity. PLoS Genet 11:e1005156
20. Couturier E, Rocha E (2006) Replication-associated gene dosage effects shape the genomes of fast-growing bacteria but only for transcription and translation genes. Mol Microbiol 59:1506–1518
21. Sun L, Alexander HK, Bogos B et al (2018) Effective polyploidy causes phenotypic delay and influences bacterial evolvability. PLoS Biol 16:e2004644
22. Espeli O, Mercier R, Boccard F (2008) DNA dynamics vary according to macrodomain topography in the *E. coli* chromosome. Mol Microbiol 68:1418–1427
23. Li Y, Austin S (2002) The P1 plasmid is segregated to daughter cells by a 'capture and ejection' mechanism coordinated with *Escherichia coli* cell division. Mol Microbiol 46:63–74
24. Aedo SJ, Orman MA, Brynildsen MP (2019) Stationary phase persister formation in *Escherichia coli* can be suppressed by piperacillin and PBP3 inhibition. BMC Microbiol 19:140
25. Ferullo DJ, Lovett ST (2008) The stringent response and cell cycle arrest in *Escherichia coli*. PLoS Genet 4:e1000300
26. Ferullo DJ, Cooper DL, Moore HR, Lovett ST (2009) Cell cycle synchronization of *E. coli* using the stringent response, with fluorescence labeling assays for DNA content and replication. Methods 48:8–13
27. Marie D, Vaulot D, Partensky F (1996) Application of the novel nucleic acid dyes YOYO-1, YO-PRO-1, and PicoGreen for flow cytometric analysis of marine prokaryotes. Appl Environ Microbiol 62:1649–1655
28. Hoechst 33342, trihydrochloride, trihydrate—10 mg/mL solution in water. https://www.thermofisher.com/order/catalog/product/H3570. Accessed 20 Nov 2020
29. Nucleic acid stains—section 8.1—US. https://www.thermofisher.com/us/en/home/references/molecular-probes-the-handbook/nucleic-acid-detection-and-genomics-technology/nucleic-acid-stains.html. Accessed 20 Nov 2020
30. Little D, Luft C, Mosaku O et al (2019) High-content analysis of mitochondrial function in iPSC-derived neurons. In: Mandenius C-F, Ross JA (eds) Cell-based assays using iPSCs for drug development and testing. Springer, New York, NY
31. Lee D, Ko W-K, Hwang D-S et al (2016) Use of baicalin-conjugated gold nanoparticles for apoptotic induction of breast cancer cells. Nanoscale Res Lett 11:381
32. Wang R, Dang Y-L, Zheng R et al (2014) Live cell imaging of *in vitro* human trophoblast syncytialization. Biol Reprod 90:117
33. Belloc F, Dumain P, Boisseau MR et al (1994) A flow cytometric method using Hoechst 33342 and propidium iodide for simultaneous

cell cycle analysis and apoptosis determination in unfixed cells. Cytometry 17:59–65

34. Rice GC, Gray JW, Dean PN, Dewey WC (1984) Fluorescence-activated cell sorting analysis of the induction and expression of acute thermal tolerance within the cell cycle. Cancer Res 44:2368–2376
35. Srivastava VK, Nalbantoglu J (2008) Flow cytometric characterization of the DAOY medulloblastoma cell line for the cancer stem-like phenotype. Cytometry A 73A:940–948
36. Blaheta RA, Kronenberger B, Woitaschek D et al (1998) Development of an ultrasensitive *in vitro* assay to monitor growth of primary cell cultures with reduced mitotic activity. J Immunol Methods 211:159–169
37. van Zandvoort MAMJ, de Grauw CJ, Gerritsen HC et al (2002) Discrimination of DNA and RNA in cells by a vital fluorescent probe: lifetime imaging of SYTO13 in healthy and apoptotic cells. Cytometry 47:226–235
38. Knowles RB, Sabry JH, Martone ME et al (1996) Translocation of RNA granules in living neurons. J Neurosci 16:7812–7820
39. Poot M, Gibson LL, Singer VL (1997) Detection of apoptosis in live cells by MitoTracker™ Red CMXRos and SYTO dye flow cytometry. Cytometry 27:358–364
40. Wlodkowic D, Skommer J, Pelkonen J (2007) Towards an understanding of apoptosis detection by SYTO dyes. Cytometry A 71A:61–72
41. Guindulain T, Comas J, Vives-Rego J (1997) Use of nucleic acid dyes SYTO-13, TOTO-1, and YOYO-1 in the study of *Escherichia coli* and marine prokaryotic populations by flow cytometry. Appl Environ Microbiol 63:4608–4611
42. Hirons GT, Fawcett JJ, Crissman HA (1994) TOTO and YOYO: new very bright fluorochromes for DNA content analyses by flow cytometry. Cytometry 15:129–140
43. Goormaghtigh F, Melderen LV (2019) Single-cell imaging and characterization of *Escherichia coli* persister cells to ofloxacin in exponential cultures. Sci Adv 5:eaav9462
44. Wery M, Woldringh CL, Rouviere-Yaniv J (2001) HU-GFP and DAPI co-localize on the *Escherichia coli* nucleoid. Biochimie 83:193–200
45. Wiegand I, Hilpert K, Hancock REW (2008) Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 3:163–175

Part IV

Omics Approaches in Persistence Research

Chapter 10

Analyzing Persister Proteomes with SILAC and Label-Free Methods

Bork A. Berghoff

Abstract

State-of-the-art mass spectrometry enables in-depth analysis of proteomes in virtually all organisms. This chapter describes methods for the analysis of persister proteomes by mass spectrometry. Stable isotope labeling by amino acids in cell culture (SILAC) is applied to assess protein biosynthesis in persister cells, which are isolated by treatment with beta-lactam antibiotics. Furthermore, persister proteomes during the postantibiotic recovery phase are analyzed by label-free quantification. The presented methods are valuable tools to shed light on persister physiology.

Key words Proteomics, Mass spectrometry, SILAC, Label-free quantification, Persister cells, Postantibiotic recovery

1 Introduction

Persister cells are in a transient state of reduced cellular activity, which allows them to withstand antibiotic treatments for an extended period compared to the actively growing part of the population. Assessing persister physiology is key to understanding which factors determine the persister state. Persister physiology may be studied at several levels, such as morphology, metabolic state and gene expression. High-throughput methods provide global information on molecules with changed abundances, and have been proven useful to study persister subpopulations. An important step for high-throughput analyses is isolation of persister fractions, which can be achieved by fluorescence-activated cell sorting (FACS) of reporter strains [1, 2]. Alternatively, persister fractions can be isolated by elimination of nonpersisters using beta-lactam antibiotics during exponential phase [3], which is considered as a simple and convenient method. However, wild-type cultures suffer from low persister levels in exponential phase, which complicates persister isolation and high-throughput analyses.

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Controllable expression of toxins or administration of bacteriostatic agents can be applied to induce the persister state and to increase the size of persister fractions [4, 5]. Alternatively, mutants with a "high persistence" (hip) phenotype are ideal model systems to apply high-throughput methods [3].

High-throughput proteomics by mass spectrometry (MS) analysis is a powerful tool, but has rarely been addressed to study persister physiology in planktonic cultures [6]. However, during the last years this gap was closed by several studies, using different persister enrichment and isolation methods [7–10].

The methods described here were used to assess the persister proteome of a high persistence mutant in *Escherichia coli* [11]. The type I toxin–antitoxin system TisB/IstR-1 is involved in persister formation upon DNA damage stress [12]. TisB is a small protein that targets the inner membrane, causing depolarization and ATP depletion [13, 14]. Deregulation of toxin TisB by deletion of regulatory RNA elements produced mutant Δ1–41 Δ*istR*, which has an enriched persister fraction during exponential phase [15]. Persister cells were isolated by treatment with the beta-lactam antibiotic ampicillin during exponential phase. In the Methods section, two different protocols for analysis of persister proteomes will be described: (1) analysis of protein biosynthesis in persister cells by metabolic labeling using stable isotope amino acids (SILAC), and (2) analysis of the persister proteome during post-antibiotic recovery by label-free quantification.

SILAC was originally developed for mammalian cell lines [16], but has successfully been applied to several microorganisms [17–20]. Importantly, auxotrophic strains are not mandatory for bacterial SILAC experiments, since incorporation of stable isotope-containing amino acids is sufficiently high (≥92%) also in prototrophic strains [17, 18]. In a pulsed-SILAC approach, the stable isotope amino acid L-lysine-$^{13}C_6$,$^{15}N_2$ (Lys-8) was administered in parallel to the ampicillin treatment during exponential phase (Fig. 1a). Using liquid chromatography (LC)-MS/MS analysis, protein biosynthesis in persister cells was assessed by Lys-8 incorporation into proteins. In exponential cultures without ampicillin, Lys-8 incorporation was on average 71.9% after 4 h of incubation (Fig. 1b). In persister fractions, isolated by ampicillin, Lys-8 incorporation was on average 33.6%, demonstrating that persister cells have a reduced translational activity and/or protein turnover (Fig. 1c). Using Significance B analysis [21], several proteins were identified in the persister fraction with a Lys-8 incorporation significantly higher than the average of proteins (Fig. 1d). These proteins are likely physiological markers of persister cells, as exemplified by the master regulator of the general stress response, RpoS, and several RpoS-dependent proteins [11].

Another intriguing question in the persister field concerns persister awakening and postantibiotic recovery. Isolated persister fractions from the high persistence mutant Δ1–41 Δ*istR* were

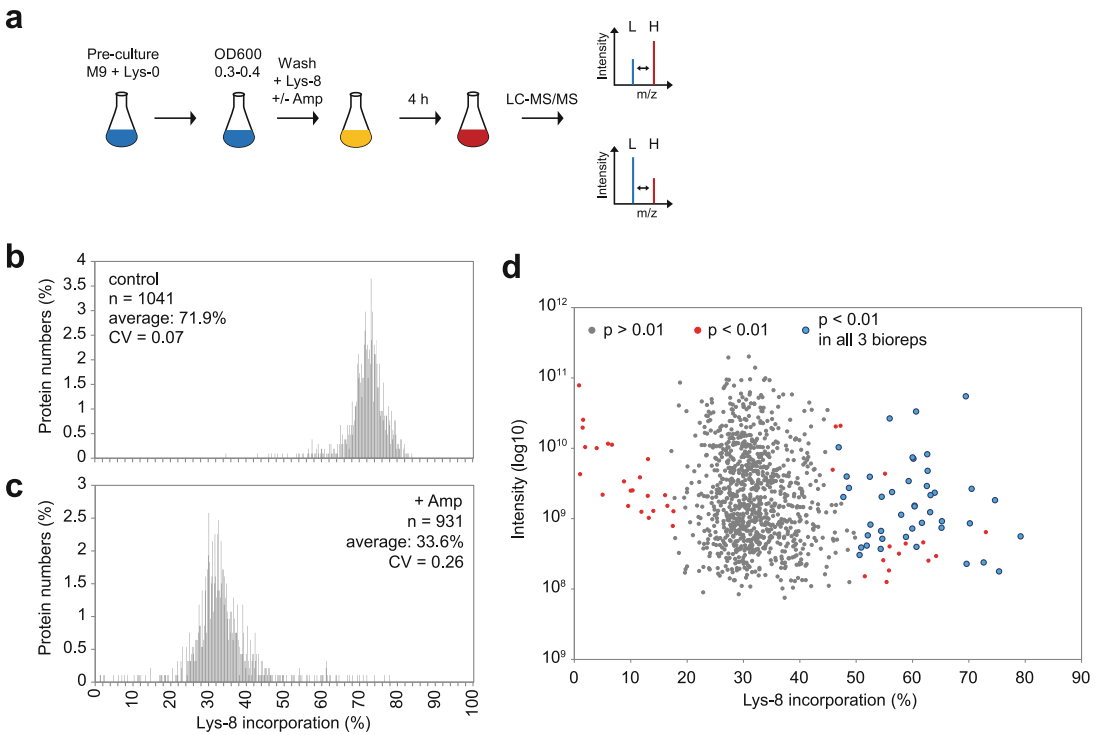

Fig. 1 Pulsed-SILAC and LC-MS/MS to assess protein biosynthesis in persister cells. (**a**) Experimental workflow of the pulsed-SILAC approach in M9 minimal medium. Unlabeled L-lysine (Lys-0) is used for precultures. At exponential phase, cells are washed and pulse-labeled with Lys-8 in the presence of ampicillin (+ Amp) for 4 h. A Lys-8-treated culture without ampicillin is used as labeling control. Proteins are analyzed by LC-MS/MS. Heavy (H) to light (L) protein ratios are calculated to assess Lys-8 incorporation. (**b**, **c**) Histograms of Lys-8 incorporation (%). The number (*n*) of analyzed proteins, the average Lys-8 incorporation, and the coefficient of variation (CV) are indicated. (**d**) Significance B analysis of Lys-8 incorporation in a representative experiment. Biological triplicates were used to identify proteins with a significantly increased biosynthesis in persister cells (blue dots)

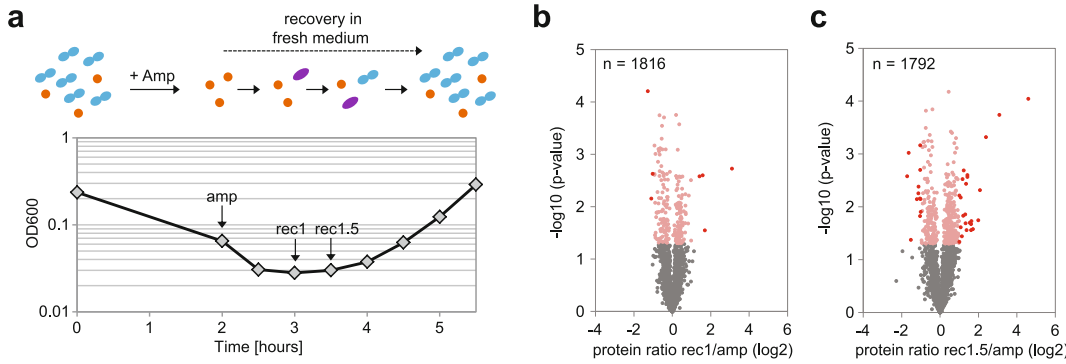

Fig. 2 Proteome analysis of postantibiotic recovery using LC-MS/MS and label-free quantification. (**a**) Top: Illustration of a recovery experiment. Cultures are treated with ampicillin (+ Amp) to lyse growing cells (blue) and to isolate persister cells (orange). Persister cells are washed and transferred to fresh medium to allow recovery (purple). Bottom: Representative growth curve of a recovery experiment in LB medium. An ampicillin-treated sample (amp) and recovery samples (rec1 and rec1.5) are withdrawn at the indicated time points, followed by LC-MS/MS analysis and label-free quantification. (**b**, **c**) Volcano plots illustrating *p*-values ($-\log_{10}$) versus protein ratios ($\log_2$) as determined by label-free quantification. The number (*n*) of quantified proteins is indicated. Proteins with significant changes during the recovery phase are highlighted as red dots

transferred to fresh medium to allow awakening and recovery of persister cells (Fig. 2a). Persister proteomes were compared at different time points during the recovery phase using LC-MS/MS and label-free quantification (Fig. 2b, c), which identified proteins with importance to the recovery process [11].

We propose that the methods presented here will be useful to study persister proteomes in many model bacteria. Accumulation of proteome data from different bacterial persister fractions, and their comparison, might reveal universal persistence factors. This in turn will provide means for tackling persister cells in clinical settings.

2 Materials

2.1 Bacterial Strains

The methods described here were applied to study the persister proteome of the high persistence mutant Δ1–41 Δ*istR* [11]. This mutant is derived from *E. coli* K-12 wild type MG1655 and has up to 100-fold more persister cells during exponential phase than the wild type when treated with the beta-lactam antibiotic ampicillin or the fluoroquinolone antibiotic ciprofloxacin [15]. High persister levels (>1%) are beneficial for proteome analysis, since they simplify the persister isolation step and enable deep proteome profiling.

2.2 Media and Reagents

For SILAC, minimal media are preferable (*see* **Note 1**). For label-free methods, any medium composition might be used. Here, we used M9 minimal medium and lysogeny broth (LB) for cultivation of *E. coli*.

1. LB medium (Miller): Mix 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 1 L distilled water and autoclave.
2. M9 salts (5× stock solution): Dissolve 64 g $Na_2HPO_4{\cdot}7H_2O$, 15 g KH_2PO_4, 2.5 g NaCl, and 5 g NH_4Cl in 1 L distilled water and autoclave.
3. 1 M $MgSO_4$ solution: Dissolve 24.65 g $MgSO_4{\cdot}7H_2O$ in 100 mL distilled water and autoclave.
4. 1 M $CaCl_2$ solution: Dissolve 11.1 g $CaCl_2$ in 100 mL distilled water and autoclave.
5. 1 mg/mL thiamine solution: Dissolve 10 mg thiamine hydrochloride in 10 mL distilled water. Sterilize by filtration (0.22 μm), prepare 1 mL aliquots and store at −20 °C.
6. 20% (w/v) glucose solution: Dissolve 20 g glucose in 100 mL distilled water and autoclave.
7. M9 minimal medium: Mix 777 mL water, 200 mL of M9 salts (5×), 20 mL of 20% (w/v) glucose, 2 mL of 1 M $MgSO_4$, 1 mL of 1 mg/mL thiamine, and 100 μL of 1 M $CaCl_2$. $CaCl_2$ should be added last to avoid precipitation.
8. 10 mg/mL L-lysine solution: Dissolve 10 mg regular L-lysine (Lys-0) or L-lysine-$^{13}C_6$,$^{15}N_2$ (Lys-8; *see* **Note 2**) in 1 mL distilled water, sterilize by filtration (0.22 μm) and store at 4 °C.
9. 100 mg/mL ampicillin solution: Dissolve 1 g ampicillin sodium salt in 10 mL distilled water. Sterilize by filtration (0.22 μm), prepare 1 mL aliquots and store at −20 °C (*see* **Note 3**).
10. 0.9% (w/v) NaCl solution: Dissolve 9 g NaCl in 1 L distilled water and autoclave.

2.2.1 Protein Sample Preparation

1. Sodium dodecyl sulfate (SDS) lysis buffer: Dissolve 4 g SDS in 90 mL distilled water and add 10 mL of 1 M Tris–HCl (pH 7.6). Store at room temperature.
2. Urea buffer: Dissolve 36 g urea and 15.2 g thiourea in 90 mL distilled water and add 1 mL of 1 M HEPES buffer. Check the pH and, if necessary, adjust to a final pH of 8. Fill up with distilled water to reach a final volume of 100 mL. Prepare 10 mL aliquots and store at −20 °C.
3. 1 M dithiothreitol solution: Dissolve 1.54 g dithiothreitol in 10 mL distilled water. Prepare 1 mL aliquots and store at −20 °C.
4. 550 mM iodoacetamide solution: Dissolve 101.7 mg iodoacetamide in 1 mL distilled water. Prepare fresh and store in the dark until use.

5. 0.5 μg/μL Lys-C: Add the desired amount of distilled water to the vial supplied by a company. For example, add 100 μL distilled water to 50 μg Lys-C. Prepare 10 μL aliquots and store at −20 °C.
6. 0.5 μg/μL trypsin: Add the desired amount of distilled water to the vial supplied by a company. For example, add 100 μL distilled water to 50 μg trypsin. Prepare 10 μL aliquots and store at −20 °C.

3 Methods

3.1 Pulsed-SILAC of Persister Cells

1. Add 5 mL of M9 minimal medium to a 50-mL Erlenmeyer flask and add 15 μL of 10 mg/mL Lys-0. The final concentration of L-lysine is 30 μg/mL. Inoculate a single colony and grow the culture overnight (~16 h) at 37 °C with shaking (180 rpm). Cells will adapt to L-lysine utilization due to the presence of Lys-0 during overnight incubation. Use appropriate selection markers depending on the strain.
2. The next day, add 10 mL of M9 minimal medium to a 100-mL Erlenmeyer flask, add 30 μL of 10 mg/mL Lys-0 and 100 μL overnight culture (dilution of 1:100). Grow the culture at 37 °C with shaking (180 rpm) until exponential phase (OD_{600} of 0.3–0.4) is reached.
3. Transfer the culture to a centrifugation tube and pellet cells by centrifugation (10,000 × *g*, 3 min, room temperature). Resuspend the cell pellet in 1 mL of M9 minimal medium (without L-lysine) and transfer to a 1.5-mL reaction tube. After centrifugation in a microcentrifuge (10,000 × *g*, 3 min, room temperature), resuspend the cell pellet in 1 mL of M9 minimal medium (without L-lysine). These washing steps will remove Lys-0.
4. Add 1 mL of resuspended cells to a 100-mL Erlenmeyer flask, containing 9 mL of M9 minimal medium, 30 μL of 10 mg/mL Lys-8 and 20 μL of 100 mg/mL ampicillin. Incubate at 37 °C with shaking (180 rpm) for 4 h (*see* **Note 4**).
5. As a control, prepare and incubate a culture as described in **step 4**, but omit ampicillin.
6. Transfer the cultures from **steps 4** and **5** to a centrifugation tube and pellet cells by centrifugation (10,000 × *g*, 3 min, 4 °C; *see* **Note 5**). Resuspend each cell pellet in 1 mL of ice-cold M9 minimal medium (without L-lysine) and transfer to a 1.5-mL reaction tube. After centrifugation in a microcentrifuge (10,000 × *g*, 3 min, 4 °C), cell pellets can be stored at −20 °C until sample preparation for MS analysis.

3.2 Persister Recovery

1. Add 5 mL of LB medium to a 50-mL Erlenmeyer flask, inoculate a single colony and grow the culture overnight (~16 h) at 37 °C with shaking (180 rpm). Use appropriate selection markers depending on the strain.
2. The next day, add 20 mL of LB medium to a 200-mL Erlenmeyer flask and add 200 μL of overnight culture (dilution of 1:100). Grow culture at 37 °C with shaking (180 rpm) until exponential phase (OD_{600} of 0.4–0.5) is reached.
3. Add 40 μL of 100 mg/mL ampicillin and incubate at 37 °C with shaking (180 rpm) for 2 h (*see* **Note 4**).
4. For ampicillin-treatment samples (*see* Fig. 2), transfer the culture from **step 3** to a centrifugation tube and pellet the cells by centrifugation (10,000 × *g*, 3 min, 4 °C; *see* **Note 5**). Resuspend the cell pellet in 1 mL of ice-cold 0.9% NaCl and transfer to a 1.5-mL reaction tube. After centrifugation in a microcentrifuge (10,000 × *g*, 3 min, 4 °C), cell pellets can be stored at −20 °C until sample preparation for MS analysis.
5. For recovery samples (*see* Fig. 2), transfer the culture from **step 3** to a centrifugation tube and pellet cells by centrifugation (10,000 × *g*, 3 min, room temperature). Resuspend the cell pellet in 1 mL of 0.9% NaCl and transfer to a 1.5-mL reaction tube. After centrifugation in a microcentrifuge (10,000 × *g*, 3 min, room temperature), resuspend the cell pellet in 1 mL fresh LB medium and add to a 200-mL Erlenmeyer flask, containing 19 mL LB medium.
6. Incubate the culture from **step 5** at 37 °C with shaking (180 rpm) to allow recovery of persister cells. After an appropriate time (*see* **Note 6**), harvest the cells as explained in **step 4**. Cell pellets can be stored at −20 °C until sample preparation for MS analysis.

3.3 Sample Preparation for MS

1. Resuspend the cell pellet in 20–40 μL of SDS lysis buffer (*see* **Note 7**). Incubate for 10 min at 70 °C and sonicate using a sonotrode (10 pulses at 20% output intensity for 1 s). This procedure will lyse the cells and homogenize the sample.
2. Remove cell debris by centrifugation in a microcentrifuge (16,000 × *g*, 10 min, room temperature; *see* **Note 8**). Solubilized proteins are now in the supernatant.
3. Transfer the supernatant to a 1.5-mL reaction tube and determine the protein concentration (*see* **Note 9**).
4. Transfer ~10 μg protein to a new 1.5-mL reaction tube (*see* **Note 10**).
5. Add 4 volumes of acetone (100%) and incubate at −20 °C for 1 h to precipitate proteins. Centrifuge in a microcentrifuge (14,000 × *g*, 10 min, 4 °C) to pellet proteins. Add 100 μL

acetone (90%), vortex gently and repeat the centrifugation step. Open the lid of the reaction tube and dry the protein pellet at room temperature (e.g., in a fume hood; *see* **Note 11**).

6. Add 20 μL urea buffer to the protein pellet and resolve by gentle pipetting (*see* **Note 12**).
7. Add 10 mM dithiothreitol and incubate at room temperature for 30 min. Then, add 55 mM iodoacetamide and incubate at room temperature for 20 min in the dark. These steps will reduce disulfide bonds and alkylate sulfhydryl groups of cysteine residues.
8. Enzymatic cleavage of proteins (in-solution digest) is performed in a two-step protocol. First, Lys-C is added in a 100:1 protein-to-enzyme ratio, and reactions are incubated for 3 h at room temperature. Next, trypsin is added in a 100:1 protein-to-enzyme ratio, and reactions are incubated overnight at room temperature.
9. The resulting peptides are desalted using stop and go extraction (STAGE) tips (*see* **Note 13**).

3.4 MS Analysis

Detailed information on how LC-MS/MS analysis is performed would exceed the scope of this chapter. For analysis of the high persistence mutant Δ1–41 Δ*istR*, an UHPLC system (EASY-nLC 1000, Thermo Fisher Scientific) and a QExactive HF Orbitrap mass spectrometer (Thermo Fisher Scientific) were applied [11]. We would like to refer the reader to a detailed description of technical specifications for LC-MS/MS [22]. For processing of MS raw data, we recommend the MaxQuant software package and the implemented Andromeda search engine [21]. For statistical analysis, including Significance B analysis, we recommend the Perseus software platform [23].

4 Notes

1. For SILAC experiments, minimal media should not contain the specific amino acid that is used for metabolic labeling of proteins. Unlabeled amino acids in the medium will compete with stable isotope amino acids for incorporation into proteins, which will impair downstream analysis and quantification. In general, there is no need for using strains that are auxotrophic for the respective amino acid. If minimal media are used, incorporation rates of stable isotope amino acids are satisfying also in prototrophic strains [11, 17, 18].
2. Here, we used L-lysine-$^{13}C_6$,$^{15}N_2$ (Lys-8) for metabolic labeling of proteins. However, several alternatives exist, including L-lysine-$^{13}C_6$ (Lys-6) and L-arginine-$^{13}C_6$ (Arg-6). Furthermore, combinations of L-lysine and L-arginine isotopes can be

applied. Some MS facilities might prefer distinct isotopes for downstream quantification processes, and we therefore strongly recommend consulting the cooperating MS facility before deciding on a stable-isotope amino acid.

3. Stability of ampicillin depends on temperature and pH. For long-term storage, we recommend to freeze aliquots at −20 °C. Avoid extensive freeze–thaw cycles. Ideally, use a fresh aliquot for every experiments.
4. We used a final ampicillin concentration of 200 μg/mL, which is approximately 20× the minimum inhibitory concentration (MIC) for *E. coli* MG1655. Note that ampicillin at this concentration efficiently lyses growing cells, while nongrowing cells (e.g., persister cells) are largely recalcitrant. In stationary phase, almost all cells are in a nongrowing state and tolerate ampicillin. Hence, major cell lysis will not be observed in stationary-phase cultures. By contrast, in exponential-phase cultures, the effectiveness of ampicillin can be easily recognized by a significant drop in optical density (*see* Fig. 2a), since the majority of cells is in a growing state. Clearance of growing cells depends on the doubling time, which in turn depends on the medium composition. Hence, the ampicillin treatment duration needs to be adapted to the growth medium. In LB medium, the doubling time for *E. coli* MG1655 is 25–30 min, and major lysis is observed after 30 min. In M9 minimal medium, the doubling time is approximately 60 min, and major lysis is observed after 60 min. We recommend to use an ampicillin treatment duration that is four times longer than the doubling time to ensure complete lysis of nonpersisters. Note that other beta-lactam antibiotics may be used as well. For example, if very long treatment durations (i.e., several days) are mandatory, ampicillin can be replaced by carbenicillin, which is more stable than ampicillin.
5. Even though long ampicillin treatment will efficiently lyse all nonpersisters and clear the lysate [3], persister isolates after centrifugation at 10,000 × *g* might suffer from contamination with cell debris. In case contamination with cell debris is a major problem, further purification steps (e.g., filtration) should be applied.
6. In order to determine the time points for recovery samples, growth curves should be consulted. For recovery experiments, persister cells are isolated using ampicillin treatment, followed by washing steps and transfer to fresh medium. This procedure will result in a typical recovery phase, in which no bulk growth resumption is detectable by optical density measurements (*see* Fig. 2a). Our results indicate that proteome analysis of samples, withdrawn at the end of the recovery phase, provides the best information on protein regulation in recovering persister cells (compare Fig. 2b, c).

7. The volume of the lysis buffer is adjusted to the number of isolated persister cells. We used 20 μL lysis buffer for ~4×10^7 persister cells. If a regular sonotrode is used for sonication, the minimum volume is 20 μL lysis buffer.
8. The centrifugation temperature should not fall below 18 °C to avoid precipitation of SDS. Precipitation of SDS might result in renaturation of proteins and reactivation of proteases.
9. We used the DC protein assay (Bio-Rad) for samples with a protein concentration of >0.5 μg/μL. If the protein concentration is <0.5 μg/μL, we recommend the Pierce 660 nm protein assay (Thermo Scientific).
10. For label-free quantification, it is essential to use equal protein amounts for each sample. GlycoBlue might also be added at this step to enable convenient visualization of protein pellets after precipitation.
11. Drying of protein pellets in a SpeedVac is not recommended. Very dry pellets are difficult to resolve in urea buffer.
12. Gentle pipetting is recommended for resolving protein pellets. Rapid pipetting or vortexing will result in foaming. Samples should not be heated to resolve protein pellets. Heat will result in decomposition of urea and inadvertent protein carbamylation.
13. STAGE tips can be fabricated according to a published protocol [24]. Alternatively, STAGE tips can be purchased (e.g., Pierce C18 Spin Tips, Thermo Scientific). Please adhere to the maximum binding capacity and the maximum loading volume when using STAGE tips.

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References

1. Rowe SE, Conlon BP, Keren I, Lewis K (2016) Persisters: methods for isolation and identifying contributing factors—a review. Methods Mol Biol 1333:17–28
2. Shah D, Zhang Z, Khodursky A et al (2006) Persisters: a distinct physiological state of *E. coli*. BMC Microbiol 6:53
3. Keren I, Shah D, Spoering A et al (2004) Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. J Bacteriol 186:8172–8180
4. Kwan BW, Valenta JA, Benedik MJ, Wood TK (2013) Arrested protein synthesis increases persister-like cell formation. Antimicrob Agents Chemother 57:1468–1473
5. Mok WWK, Park JO, Rabinowitz JD, Brynildsen MP (2015) RNA futile cycling in model

persisters derived from MazF accumulation. mBio 6:e01588–e01515

6. Sulaiman JE, Lam H (2019) Application of proteomics in studying bacterial persistence. Expert Rev Proteomics 16:227–239
7. Sulaiman JE, Hao C, Lam H (2018) Specific enrichment and proteomics analysis of *Escherichia coli* persisters from rifampin pretreatment. J Proteome Res 17:3984–3996
8. Sulaiman JE, Lam H (2020) Proteomic investigation of tolerant *Escherichia coli* populations from cyclic antibiotic treatment. J Proteome Res 19:900–913
9. Radzikowski JL, Vedelaar S, Siegel D et al (2016) Bacterial persistence is an active σ^S stress response to metabolic flux limitation. Mol Syst Biol 12:882
10. Li P, Fung Y-ME, Yin X et al (2018) Controlled cellular redox, repressive hemin utilization and adaptive stress responses are crucial to metronidazole tolerance of *Porphyromonas gingivalis* persisters. J Clin Periodontol 45:1211–1221
11. Spanka D-T, Konzer A, Edelmann D, Berghoff BA (2019) High-throughput proteomics identifies proteins with importance to postantibiotic recovery in depolarized persister cells. Front Microbiol 10:378
12. Dörr T, Vulic M, Lewis K (2010) Ciprofloxacin causes persister formation by inducing the TisB toxin in *Escherichia coli*. PLoS Biol 8:e1000317
13. Unoson C, Wagner EGH (2008) A small SOS-induced toxin is targeted against the inner membrane in *Escherichia coli*. Mol Microbiol 70:258–270
14. Gurnev PA, Ortenberg R, Dörr T et al (2012) Persister-promoting bacterial toxin TisB produces anion-selective pores in planar lipid bilayers. FEBS Lett 586:2529–2534
15. Berghoff BA, Hoekzema M, Aulbach L, Wagner EGH (2017) Two regulatory RNA elements affect TisB-dependent depolarization and persister formation. Mol Microbiol 103:1020–1033
16. Ong S-E, Blagoev B, Kratchmarova I et al (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol Cell Proteomics 1:376–386
17. Fröhlich F, Christiano R, Walther TC (2013) Native SILAC: metabolic labeling of proteins in prototroph microorganisms based on lysine synthesis regulation. Mol Cell Proteomics 12:1995–2005
18. Berghoff BA, Konzer A, Mank NN et al (2013) Integrative "omics"-approach discovers dynamic and regulatory features of bacterial stress responses. PLoS Genet 9:e1003576
19. Soufi B, Macek B (2014) Stable isotope labeling by amino acids applied to bacterial cell culture. Methods Mol Biol 1188:9–22
20. de Godoy LMF, Olsen JV, Cox J et al (2008) Comprehensive mass-spectrometry-based proteome quantification of haploid versus diploid yeast. Nature 455:1251–1254
21. Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol 26:1367–1372
22. Worzfeld T, Finkernagel F, Reinartz S et al (2018) Proteotranscriptomics reveal signaling networks in the ovarian cancer microenvironment. Mol Cell Proteomics 17:270–289
23. Tyanova S, Temu T, Sinitcyn P et al (2016) The perseus computational platform for comprehensive analysis of (prote)omics data. Nat Methods 13:731–740
24. Rappsilber J, Ishihama Y, Mann M (2003) Stop And Go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. Anal Chem 75:663–670

Chapter 11

Defining Proteomic Signatures to Predict Multidrug Persistence in *Pseudomonas aeruginosa*

Pablo Manfredi, Isabella Santi, Enea Maffei, Emmanuelle Lezan, Alexander Schmidt, and Urs Jenal

Abstract

Bacterial persisters are difficult to eradicate because of their ability to survive prolonged exposure to a range of different antibiotics. Because they often represent small subpopulations of otherwise drug-sensitive bacterial populations, studying their physiological state and antibiotic stress response remains challenging. Sorting and enrichment procedures of persister fractions introduce experimental biases limiting the significance of follow-up molecular analyses. In contrast, proteome analysis of entire bacterial populations is highly sensitive and reproducible and can be employed to explore the persistence potential of a given strain or isolate. Here, we summarize methodology to generate proteomic signatures of persistent *Pseudomonas aeruginosa* isolates with variable fractions of persisters. This includes proteome sample preparation, mass spectrometry analysis, and an adaptable machine learning regression pipeline. We show that this generic method can determine a common proteomic signature of persistence among different *P. aeruginosa* hyperpersister mutants. We propose that this approach can be used as diagnostic tool to gauge antimicrobial persistence of clinical isolates.

Keywords Persistence, Proteomic signature, Machine learning regression, *Pseudomonas aeruginosa*, Mass spectrometry

1 Introduction

The widespread use of antibiotics promotes the evolution and dissemination of resistance mechanisms. While resistant bacteria are able to grow in the presence of antibiotics, drug tolerance enables sensitive bacteria to survive during prolonged antibiotic treatment. Tolerance either pertains to all cells of a bacterial population or is limited to a subpopulation called persisters [1]. Although challenging to diagnose, persistent cells have been identified in all bacterial pathogens analyzed so far [2, 3]. For example, in cystic fibrosis patients, the opportunistic lung pathogen *Pseudomonas aeruginosa* develops tolerance and resistance during lifelong chronic infections [4, 5]. The genetic basis of antibiotic

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tolerance is difficult to address due to the high genome plasticity of *P. aeruginosa* genomes during in-host microevolution and due to the potentially large panel of mutations that can boost tolerance [6, 7]. Furthermore, the study of persistence physiology is impaired by the generally small numbers of persisters in growing bacterial populations. In addition, metabolic or proteomic analyses suffer from biases introduced during sorting and enrichment steps.

Here, we outline a different procedure to characterize specific persister physiologies. We demonstrate how proteomic signatures of entire populations of *P. aeruginosa* isolates with different fractions of persisters can provide qualitative and quantitative traits describing antibiotic tolerance and persistence. For this, we use proteomic regression models that are able to explore and accurately predict persistence in different isolates. We describe an analysis strategy that is based on an orthogonal partial least square regression (oplsr) [8–11]. This machine learning method consists of a latent variable regression method that maximizes the covariance between independent variables (proteomes) and the response phenotype (persistence). We describe a step-by-step protocol for the establishment of an oplsr proteomic model, which can be applied to study persistence or any other phenotype of interest. We show that different proteomic models can successfully predict the level of persistence in different isolates. In order to enhance both the diagnostic and exploratory capabilities of the models, we define a recursive model simplification method that leads to the isolation of a minimal set of indicators of a given phenotype. As a test case, we generated the proteomic signatures of *P. aeruginosa* hyperpersisters isolated during experimental evolution with tobramycin and successfully correlated these patterns to the different levels of persistence in these isolates. Because this is a generic method, it can be applied to identify the molecular framework associated with any quantitative phenotype. A future goal is to use this approach to rapidly predict multidrug antimicrobial persistence of clinical isolates.

2 Materials

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

2.1 General Materials

1. 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 1**).
2. 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 1 month (*see* **Note 1**).

3. Sterile glass test tubes.
4. Sterile 1.5 mL and 2 mL microtubes (low binding).
5. Spectrophotometer.
6. Benchtop microcentrifuge capable of 10,000 × *g*.
7. Incubator at 37 °C capable of shaking at 170 rpm.
8. Liquid nitrogen.

2.2 Whole Cell Lysis and Protein Extraction

1. Bioruptor (Diagenode) or other microtube sonicator.
2. Microplate-compatible bicinchoninic acid (BCA) Protein Assay Kit, Reducing Agent Compatible (e.g., Thermo-Pierce).
3. Chloroacetamide.
4. Porcine trypsin.
5. 2-propanol.
6. Ammonium hydroxide solution (25% in water).
7. HPLC water.
8. Acetonitrile.
9. Formic acid.
10. 10× stock indexed retention time (iRT) peptide solution (e.g., from Biognosys).
11. Lysis buffer: 1% sodium deoxycholate, 10 mM TCEP, 100 mM Tris-HCl, pH 8.5 (adjusted with NaOH/HCl). For 50 mL: 0.5 g sodium deoxycholate, 1 mL 0.5 M TCEP, 0.606 g TRIS base.
12. Thermomixer (microtubes heating and shaking block).
13. Reducing agent compatible BCA assay (e.g., Thermo-Pierce) or similar methods for protein measurements (optional).

2.3 Sample Preparation for LC-MS/MS: Protein Digestion

1. pH test strips.
2. 1 M ammonium bicarbonate pH 8.2.
3. 0.75 M chloroacetamide (70.125 mg/mL dissolved in water).
4. 0.5μg/μL porcine trypsin stock (stored at −80 °C).
5. Stop buffer: 5% trifluoroacetic acid (Sequencing grade) in water.
6. Wash 1 buffer: 1% trifluoroacetic acid (Sequencing grade) in 2-propanol.

1. Vacuum concentrator (e.g., SpeedVac).
2. UV/microvolume spectrometer (e.g., SpectroStar Nanodrop analyzer).

2.4 Sample Preparation for LC-MS/MS: Solid-Phase Peptide Purification

3. Wash 1 buffer: 1% trifluoroacetic acid (Sequencing grade) in 2-propanol.
4. Wash 2 buffer: 0.2% trifluoroacetic acid (Sequencing grade) in water.
5. Elution buffer: 1% (v/v) ammonium hydroxide, 19% water, and 80% acetonitrile (*see* **Note 2**).
6. LC buffer A: 0.15% formic acid, 2% acetonitrile.
7. Styrene–divinylbenzene—reversed phase sulfonate cartridges (SDB-RPS) for solid-phase extraction.
8. Ultrasonication VialTweeter (or similar).

2.5 LC-MS/MS

1. LTQ-Orbitrap Elite mass spectrometer connected to an electrospray ion source and a column heater.
2. A nanoflow liquid chromatography control system equipped with a reverse phase HPLC column (75μm × 30 cm) packed with C18 resin.
3. Solvent A: 0.1% formic acid in water.
4. Solvent B: 80% acetonitrile, 0.1% formic acid, in water.

3 Methods

Perform all handlings at room temperature. For sample generation work under sterile conditions. Wear gloves when collecting bacterial pellets and for all subsequent handlings in order to minimize contaminations with human proteins.

3.1 Sample Generation

1. Streak out bacterial strains to be analyzed from a cryostock (−80 °C) onto fresh LB plates and incubate overnight at 37 °C.
2. The next day, move the plate to a dark and dry place at room temperature. Inoculate a single colony for each strain into a test tube containing 5 mL of LB and incubate overnight at 37 °C under agitation. Repeat the inoculation two more times to generate biological triplicates (*see* **Note 1**).
3. The next day, measure the optical density of the overnight cultures at 600 nm (OD600) and pellet (10,000 × *g*, 2 min) the equivalent of 1 mL at OD600 0.6 (corresponding to ca. 5×10^8 CFU/mL) (*see* **Note 3**). Remove the culture supernatant carefully, pulse-spin and remove any remaining liquid. Flash freeze the cell pellets in liquid nitrogen and store at −80 °C.

3.2 Whole Cell Lysis and Protein Extraction

1. Cool down the microtube sonicator for 30 min (use ice in absence of a dedicated cooling system).

2. Set the Thermomixer with a microtube-compatible plate at 95 °C.
3. Resuspend the bacterial pellet (Subheading 3.1, **step 3**) in 50μL of lysis buffer (*see* **Note 4**).
4. Sonicate the samples with a standard lysis program (e.g., 30 s on, 30 s off, 10 cycles = 10 min).
5. After sonication, samples should be clear. Otherwise, repeat **step 4**.
6. Pulse spin the samples to pull down possible material from the tube walls.
7. Heat the samples for 10 min at 95 °C and 300 rpm in the Thermomixer (*see* **Note 5**).
8. Allow samples to cool and spin down at 2500 × *g* for 10 s.
9. Optional (*see* **Note 6**): Save 5μL of the supernatant to perform a BCA assay (reducing agent compatible) to measure protein concentration. Follow the BCA assay protocol according to the manufacturer's recommendations. Use lysis buffer as blank.
10. Add 1μL of chloroacetamide solution and keep at 37 °C for 30 min at 500 rpm in the Thermomixer (*see* **Note 7**).
11. Spin down at 2500 × *g* for 10 s.
12. Optional (*see* **Note 6**): Wait for the BCA assay results. If protein concentrations are above 2.5μg/μL, adjust the sample to 2.5μg/μL by diluting with lysis buffer accordingly. Take an aliquot containing no more than 50μg of total protein for further analysis. Keep the rest of the samples at −20 °C as a backup.

3.3 Sample Preparation for LC-MS/MS: Protein Digestion

For successful shotgun proteome experiments, proteins need to be efficiently digested into peptides to be amenable for LC-MS analysis. This protocol builds on a sole trypsin digestion step.

1. Adjust the sample pH between 8 and 9 using 1 M ammonium bicarbonate buffer (pH 8.2), if necessary. Use pH indicator test strips.
2. Add 2μL of the trypsin stock to a final enzyme/protein ratio of 1:50, mix and digest at 37 °C overnight without shaking.
3. The next day, spin samples at 2500 × *g* for 10 s.
4. Add 50μL of 5% trifluoroacetic acid. Important: You should see a white precipitate of sodium deoxycholate. Otherwise, set the pH to be below 2 using more 5% trifluoroacetic acid.
5. Add 100μL of wash 1 buffer. The precipitate should dissolve again at least partially.
6. Vortex and spin down at 2500 × *g* for 10 s.

3.4 Sample Preparation for LC-MS/MS: Solid-Phase Peptide Purification

1. Transfer each sample to a solid-phase extraction cartridge (SDB-RPS) held in a 2 mL microtube.
2. Spin cartridge at 2000 × *g* for 4 min. Increase the force by 200 × *g* if the liquid does not penetrate completely. Discard the flow-through.
3. Load 200 μL of wash 1 buffer and spin at 2000 × *g* for 4 min. Discard the flow-through.
4. Repeat the previous step.
5. Load 200μL of wash 2 buffer and spin again (2000 × *g*, 4 min). Discard the flow-through.
6. Repeat the previous step.
7. Place the SDB-RPS cartridges into a new 2 mL microtube labeled accordingly.
8. Elute bound peptides twice with 100μL elution buffer (2 × 2000 × *g*, 2 min).
9. Concentrate eluted peptide mixture under vacuum to complete dryness (e.g., using a SpeedVac).
10. Peptides can be stored dried at −20 °C or better −80 °C for at least 6 months.
11. Dissolve peptides in 20μL of LC buffer A immediately before use. To dissolve completely, provide five short pulses with the ultrasonicator VialTweeter followed by thorough shaking at 1400 rpm for 5 min at 25 °C in a Thermomixer.
12. Determine the peptide concentration using a microvolume spectrometer analyzer and adjust samples to a final peptide concentration of 0.5μg/μL using LC buffer A.
13. Add between 10 and 20μL of sample into an LC vial and remove air bubbles by short and low force centrifugation or by pipetting.
14. Put the vials at −20 °C or −80 °C for storage.

3.5 LC-MS/MS Analysis

LC-MS/MS analysis per se will most likely be run by a specialized group or a technology platform. We include the next protocol steps for sake of completeness. However, different viable options are possible here and will depend on the equipment and IT infrastructure available at your MS proteomic service provider.

1. For each sample, subject 1μg of total peptides to LC-MS analysis using a dual pressure LTQ-Orbitrap Elite mass spectrometer connected to an electrospray ion source and a column heater set to 60 °C. Peptide separation can be carried out using an EASY nLC-1000 system equipped with a RP-HPLC column (75μm × 30 cm) packed with C18 resin for example.

2. Run the HPLC as successive linear gradients from 95% solvent A and 5% solvent B to 35% solvent B over 50 min, to 50% solvent B over 10 min, to 95% solvent B over 2 min and stay at 95% solvent B over 18 min at a flow rate of 0.2μL/min.
3. Set the data acquisition mode to obtain one high resolution MS scan in the cyclotron part of the mass spectrometer: Resolution at 120,000 full width at half maximum (400 *m/z*, MS1) followed by MS/MS (MS2) scans in the linear ion trap of the 20 most intense MS signals.
4. Enable the charged state screening modus to exclude unassigned and single-charged ions.
5. Set the dynamic exclusion duration to 30 s, the collision energy to 35%, and the number of microscans acquired for each spectrum to one.

3.6 Peptide Level Data Set Generation

1. Import the acquired "`.raw`" files into the Progenesis QI software (v2.0, Nonlinear Dynamics Limited).
2. Extract the peptide precursor ion intensities across all samples applying the default parameters. This generates "`.mgf`" files.
3. Generate a decoy database containing normal and reverse sequences of the concatenated *Homo sapiens* (for most frequent contaminant detection) and *P. aeruginosa* proteomes (e.g., from UniProt) using the SequenceReverser tool from the MaxQuant software (Version 1.0.13.13). Alternatively, use any other targeted organism proteome.
4. Search the "`.mgf`" files using MASCOT against the decoy database with the following criteria: Full tryptic specificity (cleavage after lysine or arginine residues, unless followed by proline), three missed cleavages allowed, carbamidomethylation (on cysteine) set as fixed modification, oxidation (of methionine) and protein N-terminal acetylation accepted as variable modifications, and mass tolerance of 10 ppm (precursor) and 0.6 Da (fragments).
5. Filter the database search results using the ion score to set the false discovery rate to 1% on the peptide and protein level, respectively, based on the number of reverse protein sequence hits in the datasets.
6. Generate a normalized label-free quantification using the SafeQuant R package v.2.3.4 (https://github.com/eahrne/SafeQuant/) [12] in order to obtain peptides relative abundances. Only isoform-specific peptide ion signals should be considered for quantification.

3.7 Regression and Feature Reduction of Proteomic Models of Persistence

In our test case, 42,104 different peptides could be identified and quantified. These could be unequivocally assigned to 3487 proteins. Altogether, each proteome was therefore defined by 45,591 variables with different levels of dependence (peptides and proteins).

Multivariate regression methods such as the Partial Least Squares Regression [9] were developed to deal with this many (potentially colinear) predictive variables (MS data) from only few samples [11]. This technique is therefore suited for the analysis of high-throughput analytical approaches like transcriptomics, proteomics or metabolomics [13–15].

3.7.1 Dataset Preparation

For the first steps of the analysis, use the freely accessible Perl [16] script "TabMaker.4R.pl" to gather and refine the data generated (https://github.com/Pablo-Manfredi/PLS-regression-of-MS-data) (*see* **Notes 8** and **9**). This script will compute the peptide MS signals for each protein and combine peptide and protein MS data with the phenotypic data (e.g., persistence). It also performs a sample normalization by dividing each MS signal by the total signal of the sample. The script needs to be pasted in a directory containing:

1. A two columns "`Phenotype.tsv`" file (*see* **Note 10**) with sample references in the first column and phenotype values (e.g., bacterial survival after 7 h of treatment at 16μg/mL of tobramycin for persistence) in the second. Each replicate included in the MS data set should have an associated phenotype (*see* **Note 11**). Also include a headline as first line in the file. Its content does not matter as it is skipped.
2. A "`PeptideMS.tsv`" file (*see* **Note 10**) containing peptide intensities for each individual sample with the following format (*see* **Note 12**):

```
Pep prot A.1 A.2 A.3 B.1 B.2 B.3
AAFAPQLLDYK PA2304 6829.45 6076.12 6477.13 9218.43 9464.76
7391.39
AAAAEDPFVISVK PA3810 8626.04 8873.76 8229.54
9116.45 9343.52 9272.23
GELVQSWP[5]Oxi PA2867 1439.79 1861.30 1629.01 1295.86 1706.28
1307.05
...
```

As a result, the script generates the "`Table4R.tsv`" file, which contains all the formatted information for the actual oplsr using R (*see* **Note 10**).

3.7.2 Orthogonal Partial Least Square Regression (oplsr) of MS Data

Install RStudio [17] (*see* **Note 13**) and copy the "`oPlsr-Phenot-MS.R`" R script (https://github.com/Pablo-Manfredi/PLS-regression-of-MS-data) into your working directory, that should also contain the "`Table4R.tsv`" file, and open it on RStudio. We recommend running the "`oPlsr-Phenot-MS.R`" script line by line by clicking on each line followed by pressing Ctrl+Enter. This

will increase readability of the process and ease adaptation of your data to our script frame. The different steps of the R script and corresponding lines are described here.

1. R library packages (lines 4–12): The script requires several R library packages to be installed and updated. If those libraries are not yet installed in your environment, "unhash" the corresponding lines in the header and execute them (e.g., "`install.packages("BiocManager")`"). Update all packages when asked (type "a").
2. Functions (lines 15–38): This code block defines the Variable Importance in Projection (VIP) functions required for the feature reduction (i.e., the model simplification step) (*see* **Note 14**).
3. Data preparation (lines 41–52): Because data loading can be lengthy in R, you should use the optimized data import package "`data.table`" to speed up loading time to a few seconds (*see* **Note 15**). For better performance of oplsr, you will have to standardize the MS data. However, despite a slight increase of oplsr performance when standardizing the phenotypic data, we prefer to leave this information in a "biologist friendly" form to facilitate interpretation later on. Note that this is not always recommended when variances are very heterogeneous. The two options can be tested by replacing the two "`FALSE`" values by "`TRUE`" at line 51 and testing with or without phenotype standardization. In the case of persistence, we employ log10 (survival) scores which gives phenotypes in the approximate range of 1–10.
4. Oplsr regression from the whole dataset (lines 55–62): This block performs the first regression on the whole dataset (*see* **Note 16**) using the plsr function from the pls R package [10]. It produces two graphs representing the performance of the proteomic signatures (i.e., the models) in predicting the actual phenotype (here: persistence) of each sample (*see* **Notes 16** and **17**).
   (a) The first graph (Fig. 1a) reports the overall performance (errors of prediction) of models with increasing numbers of components (i.e., increasing complexity in the data interpretation) using the whole dataset. In order to avoid overfitting and to keep a visual display of the model, we limit the number of components to 2 (*see* **Notes 18** and **19**).
   (b) The second graph displays the leave-one-out cross-validation of the selected model (2 components) (Fig. 1b) (*see* **Notes 16** and **19**).

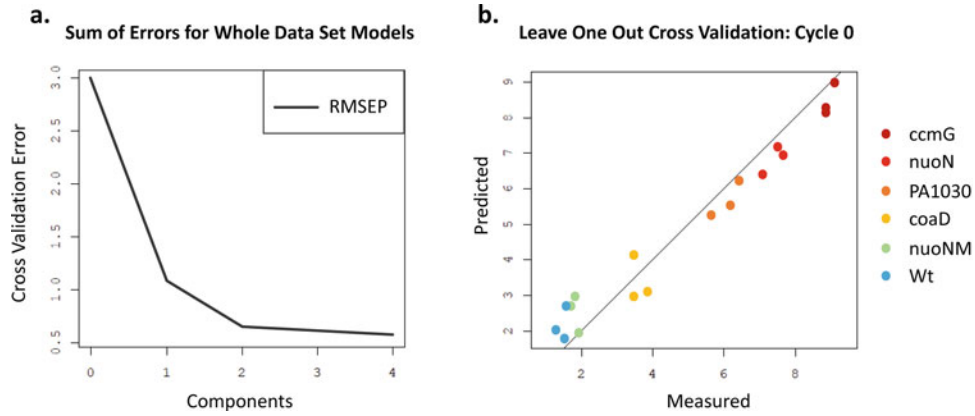

Fig. 1 Oplsr whole proteome model of *P. aeruginosa* antimicrobial persistence. Leave-one-out cross-validation of the proteomic oplsr models for tobramycin persistence. (**a**) Increasingly complex models (higher number of components, *x*-axis) fit the whole dataset (45,591 variables) while displaying decreasing root mean square error of prediction (RMSEP, *y*-axis). (**b**) Predicted (*y*-axis) and experimentally determined (*x*-axis) numbers of surviving cells after 7 h of tobramycin treatment (LOG10) for biological triplicates of six isogenic variants of *P. aeruginosa* (color beads)

5. Preparing for feature selection (lines 65–77): This allows you to set all the variables that are required in the iterative feature reduction step (*see* **Note 20**). This includes an arbitrary cutoff (=1) for the VIP, which assesses the level of contribution of a given variable (here, a peptide or a protein) to the current predicting model (here, the proteomic signature).
6. Iterative feature reduction (lines 80–118): This loop is arbitrarily set to run 15 cycles of variable reduction. Each cycle removes variables that are of least importance for the prediction of the phenotype, builds a new predictive model and repeats phenotype prediction. This process is repeated until reaching a level where too few variables are left to perform the oplsr, thus resulting in an error message. If 15 cycles are not enough, this number can be increased at line 84. During each cycle the script produces similar graphic outputs as for cycle 0 (*see* block lines 55–62) (*see* **Note 19**). In addition, two tables reporting the VIP values (`VIP_run_X.abo1.comp2.tab`) and the standardized MS signal values (`VIP_run_X7.abo1.comp2.tab.tab`) for the remaining variables at a given cycle "`X`" are printed in the working directory (*see* **Note 10** for format specifications).
7. Plot summary of the feature reduction (lines 119–142): Eventually, the feature reduction cycles are summarized in a plot (Fig. 2a). This plot tracks the error of the models and the number of variables used by the models during each cycle. Often, the models with the best predictive power are among the simplest ones (Fig. 2b, c) (*see* **Note 18**). Note that

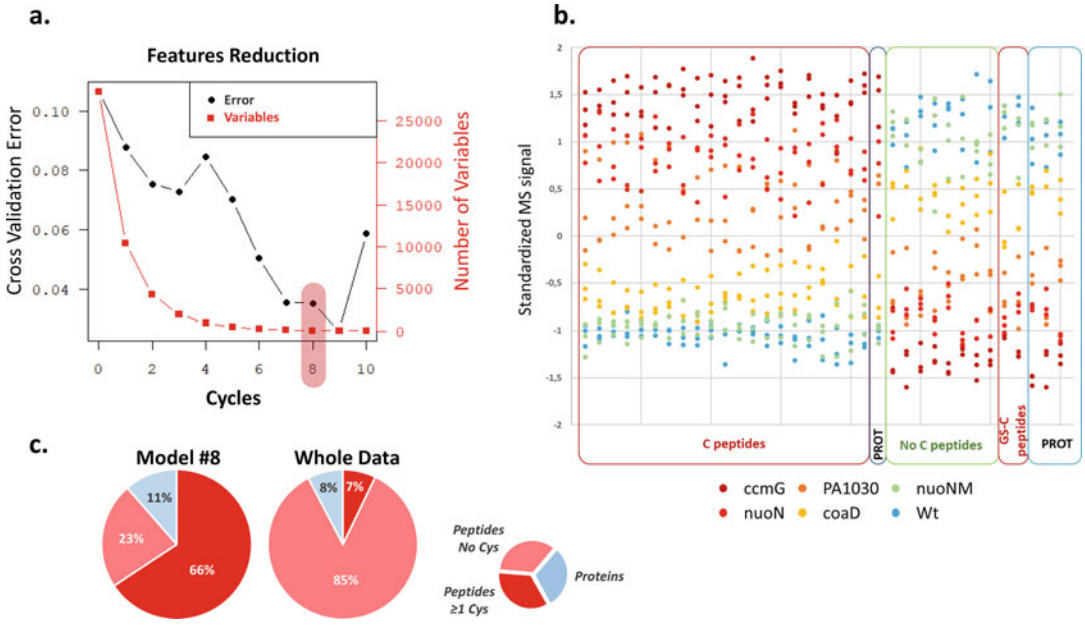

Fig. 2 Best proteomic models of antimicrobial persistence mostly contain cysteine-containing peptides. (**a**) The feature reduction process iteratively generated oplsr models integrating decreasing numbers of variables (*x*-axis). At each cycle, the overall error of prediction of models was assessed (left *y*-axis, black) and the numbers of proteomic variables used was reported (right *y*-axis, red). The best model exploiting at least 30 variables (red shade) is further explored in (**b**) and (**c**). (**b**) Standardized mass spectrometry values from different strains for best-predicting proteomic variables. The model includes four proteins and 31 peptides with or without cysteines (C). Two peptides exhibited a cysteine bound to a glutathione moiety. **(c)** Cysteine-containing peptides are significantly enriched in the optimized persistence model as compared to the initial dataset. The fraction of protein variables is depicted in blue; peptides exhibiting at least one cysteine are represented by the red fraction, peptides without cysteine residues are represented in pink

minimalistic models may be difficult to interpret in terms of biological pathways. Thus, it may be beneficial to explore models that retained a certain number of variables in order to observe clear enrichments (Fig. 2c). In our test case, peptides containing cysteines are significantly enriched as compared to the initial dataset. Cysteines are highly sensitive to oxidative modifications and thus their enrichment suggest that the oxidation state of strains is a strong indicator of persistence. If desired, you can also plot the standardized values of variables per strain to verify specific trends as predicted by the models (Fig. 2b).

4 Notes

1. Use the same LB batch preparation for the entire experiment to reduce variability due to different medium quality.

2. Caution: ammonium hydroxide solution is only 25%, not 100%!
3. At least 1µg of protein per sample is required. We recommend between 50 and 100µg (i.e., close to the maximum capacity of the solid-phase extraction step). Extracted protein amounts can be deduced from cell numbers according the following rules of thumb: Human/mouse and rat cells contain approximately 200–300 pg/cell, budding yeast 4 pg/cell, fission yeast 10 pg/cell, bacteria much less, that is, 200 fg/cell for *Escherichia coli* and 10 fg/cell for mycoplasma. In this protocol, volumes were empirically optimized for *P. aeruginosa* (100 fg/cell).
4. If the lysis buffer shows a white precipitate after thawing, mix at 50 °C, 300 rpm for 1 min in the Thermomixer.
5. During heating, microtube lids can pop open due to pressure increase. To avoid sample loss, leave lids open when placing the tubes into the Thermomix block and close them after 1 min.
6. *P. aeruginosa* samples do not require measurement of protein concentrations as sample volumes are already adapted in this protocol.
7. Chloroacetamide may precipitate during freezing. Shake for 5 min at 37 °C to dissolve any pellet.
8. Perl is a high-level and general-purpose scripting language with freely available interpreters (e.g., www.activestate.com) [16]. 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.
9. The scripts will automatically look for files placed in the same folder. To make sure that they work properly, (1) copy scripts into the same folder containing the files to analyze; and (2) keep file naming as recommended here.
10. ".tsv" stands for tabulation-separated values. These files can be created from any spreadsheet program like Excel (save as tab separated .txt file or copy/paste into an notepad-like new file). Values are accepted under most formats.
11. If the number of replicates per strain is the same, better use the data from each replicate. Otherwise, use median values per sample. Using different numbers of samples per strain in an oplsr might bias the model toward an overrepresented variant.
12. The "pep" and "prot" entries can be generated from any type of string of characters. If your dataset considers posttranslational modifications, simply concatenate this information to the peptide sequence (*see* the gray box). Adapt the sample names (A or B) at your convenience but reserve the "." character as a separator between the strain name and the replicate

number. There is no limit to the number of samples or replicates. However, there should be a perfect match between the two input files in terms of sample amount and syntax of the sample names. We advise to keep names nomenclature simple in order to reduce the chances of format incompatibilities.

13. RStudio is an open source programming environment for R programming language [17] available on Windows, Mac, and Linux (https://rstudio.com/products/rstudio/download/#download).
14. VIP [18] stands for Variable Importance in Projection. This score assesses the actual contribution of a given variable to a given oplsr model. The VIP value of a random variable will tend to be 0 while variables correlating with the phenotype will tend to display VIP scores above 1. It is generally considered that VIP values above 1 indicate an important contribution of the corresponding variable to the model. Because of the way they are computed, VIP scores change during cycles of feature reduction. During such a procedure, these scores are solely used for ranking rather than to distinguish important from unimportant variable contributions.
15. Once the data is loaded, the working environment can be saved in a more convenient "`.RData`" format for R (Session > Save Workspace As...). All intermediary variables and tables are stored in this file and can be retrieved quickly (Session > Load Workspace...) if you need to repeat the script execution.
16. The "orthogonal" variant of the classic plsr approach exploits different parts of the covariance for each linear subregression (i.e., component). In other words, the information used in the first component is not used in subsequent components. In order to avoid issues with overfitting, we generally limit the number of components to two per oplsr model.
17. Performances of models are assessed by leave-one-out cross-validation: For a given model and for each sample, a submodel excluding the given sample is computed and the phenotype of this sample is predicted. The average of differences between measured and predicted phenotypes are then used as a performance score (i.e., Root Mean Square Error of Prediction, RMSEP).
18. The requirement of additional components to reach a local minimum of the overall error (Fig. 1a) indicates that the phenotype studied is complex and implicates different molecular mechanisms. For example, proteomic models aimed at describing antibiotic resistance were unable to accurately predict MIC values with a low number of components. Besides, efficient oplsr models will be more challenging to obtain but also more biologically meaningful with increasing strain and sample numbers.

19. All graphs can be exported from RStudio as pictures by using the "Export" function on the image explorer panel. The arrow buttons on the graphics panel allow glancing over all graphs generated. In case the panel is overloaded, you can clear everything and restart the analysis using the broom icon.
20. To optimize the biological insight of persister physiology, iterative cycles of feature reduction are executed. An initial model is computed from the entire dataset and variables with a VIP above 1 are considered for the computation of the next cycle model. After each cycle, new VIP scores are computed from the new models and a variable selection is operated for VIPs above 1 for integration into the next cycle. This process greatly enhances the model predictions and best cross-validations are achieved with just few features (here: $N = 35$).

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References

1. Andersson DI, Balaban NQ, Baquero F et al (2020) Antibiotic resistance: turning evolutionary principles into clinical reality. FEMS Microbiol Rev 44:171–188
2. Fauvart M, De Groote VN, Michiels J (2011) Role of persister cells in chronic infections: clinical relevance and perspectives on anti-persister therapies. J Med Microbiol 60:699–709
3. Cohen NR, Lobritz MA, Collins JJ (2013) Microbial persistence and the road to drug resistance. Cell Host Microbe 13:632–642
4. Hansen CR, Pressler T, Hoiby N (2008) Early aggressive eradication therapy for intermittent *Pseudomonas aeruginosa* airway colonization in cystic fibrosis patients: 15 years experience. J Cyst Fibros 7:523–530
5. Santi I, Manfredi P, Maffei E, Egli A, Jenal U (2020) Evolution of antibiotic tolerance shapes resistance development in chronic *Pseudomonas aeruginosa* infections. mBio 12: e03482–e03520
6. Fridman O, Goldberg A, Ronin I et al (2014) Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. Nature 513:418–421
7. Levin BR, Concepcion-Acevedo J, Udekwu KI (2014) Persistence: a copacetic and parsimonious hypothesis for the existence of non-inherited resistance to antibiotics. Curr Opin Microbiol 21:18–21
8. Thevenot EA, Roux A, Xu Y et al (2015) Analysis of the human adult urinary metabolome variations with age, body mass index, and gender by implementing a comprehensive workflow for univariate and OPLS statistical analyses. J Proteome Res 14:3322–3335
9. Kreeger PK (2013) Using partial least squares regression to analyze cellular response data. Sci Signal 6:tr7
10. Mevik BH, Wehrens R, Liland KH, Hiemstra P (2020) R package pls: partial least squares and principal component regression. https://CRAN.R-project.org/package=pls. Accessed 19 Nov 2020
11. Martens H (2001) Reliable and relevant modelling of real world data: a personal account ofthe development of PLS regression. Chemom Intell Lab Syst 58:85–95
12. Ahrne E, Glatter T, Vigano C et al (2016) Evaluation and improvement of quantification accuracy in isobaric mass tag-based protein

quantification experiments. J Proteome Res 15:2537–2547

13. Janes KA, Albeck JG, Gaudet S et al (2005) A systems model of signaling identifies a molecular basis set for cytokine-induced apoptosis. Science 310:1646–1653
14. Kumar D, Srikanth R, Ahlfors H et al (2007) Capturing cell-fate decisions from the molecular signatures of a receptor-dependent signaling response. Mol Syst Biol 3:150
15. Kreeger PK, Mandhana R, Alford SK et al (2009) RAS mutations affect tumor necrosis factor-induced apoptosis in colon carcinoma cells via ERK-modulatory negative and positive feedback circuits along with non-ERK pathway effects. Cancer Res 69:8191–8199
16. Wall L, Christiansen T, Orwant J (2000) Programming perl, 3rd edn. O' Reilly Media, Sebastopol
17. R Core Team (2019). R: a language and environment for statistical computing. R Foundation for Statistical Computing. https://www.R-project.org/. Accessed 19 Nov 2020
18. Chong I-G, Jun C-H (2005) Performance of some variable selection methods when multicollinearity is present. Chemom Intell Lab Syst 78:103–112

Chapter 12

The Use of Experimental Evolution to Study the Response of *Pseudomonas aeruginosa* to Single or Double Antibiotic Treatment

Isabella Santi, Pablo Manfredi, and Urs Jenal

Abstract

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.

Key words Experimental evolution, Antibiotic tolerance, Adaptation, Cyclical treatment, *Pseudomonas aeruginosa*, Aminoglycosides, Fluoroquinolones

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

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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 sublineages 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 (0.22μm) 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 1 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 4000 × *g*.
8. Centrifuge for 50 mL tubes capable of 7000 × *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. 10× 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 Hypertolerant 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 4000 × *g* in the microcentrifuge and at 7000 × *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 ~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 (200μL 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 tenfold dilutions of the harvested 200μL aliquot, spot droplets of 10μL (*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 Subheading 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* Subheading 3.2).
9. Harvest an aliquot to determine CFUs. Make appropriate serial tenfold 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**).

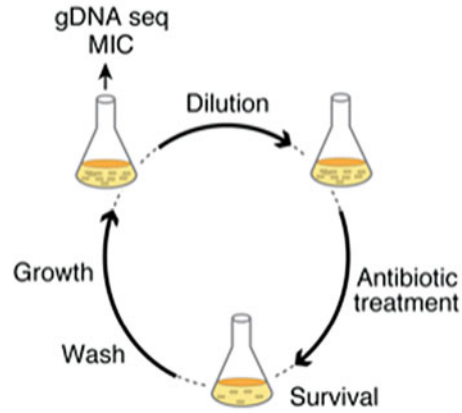

Fig. 1 Schematic of the experimental design for the iterative exposure of *P. aeruginosa* to bactericidal antibiotics

11. Go to **step 3** and repeat the procedure with daily cycles until drug resistance or tolerance has evolved (Fig. 1). 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* Subheading 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* Subheading 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 Subheadings 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. Subheading 3.2 is an adaptation from previously described methods [21].

1. Dilute the overnight culture (**step 3** of Subheading 3.1) into LB medium to reach an OD600 of 0.1.
2. Make twofold serial dilutions of the antibiotic in a 96-well plate. To do so, add 200μL of LB to column 2 and supplement it with the antibiotic to reach the double of the highest concentration to be tested. Add 100μL of LB to columns 3–10 and transfer 100μL from each column to the next, starting with column 2. Add 100μL of LB to column 11 as a control lacking

antibiotics. Add 100μL 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 Subheading 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 H_2O 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 HiSeqlane (Illumina) with a targeted coverage of about 100×.
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 Subheadings 3.3.1–3.3.3.

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
> samtools sort A.Assem.bam -o A.Assem.sort.bam

# Compile the assembly in parser readable pileup format
> samtools mpileup -A -B -x -d 1000 -f ref.fasta A.Assem.sort.
bam > A.Assem.sort.pileup
```

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:GGECGGF=G00GFGGGCGGGGGGGGGG0GG
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>GGGFGGGFGDGGGGBGG<<G>DCGGGGBGGCGGGG/GG
AE004091.2_PAO1 9	G	43	,,,,,,,,,,,,,+3ATG,,,,,,,,,,,,,,,,,	GGGGDGGGGGGECGGGGGGGEE/GG>CGGGDEGGE<GGGGCGE
AE004091.2_PAO1 10	A	43	,,	G>G@GGGGGGGD<GGGGGDC<G/GGE/GGGG/GG<EADGC8GC
AE004091.2_PAO1 11	C	43	,,	G1GG>GGGGGG0DGGGGGGEGG/FG<@EGGG@GG/GGAGA<GG
AE004091.2_PAO1 12	C	43	,,	GFEGGGGGGGGEEGGGDGG<GE<GG<G>GGGGBGGEGGGE<GG
AE004091.2_PAO1 13	G	43	....$....$...,,,$,,,,$,,,,,,,$,,,,$,,	G9CGGGGGGGG>FFGG<GG<FG/GG/GFGBGGFGGFGGG<GGE

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 fourth column of the pileup file (above):

(a) "." and "," indicate reference matches in the two DNA strands

(b) "`ATGC`" and "`atgc`" indicate substitutions

(c) insertion sequences are preceded by a "+" or a "−" sign followed by the length of the insertion or deletion (indel) and its sequence,

(d) "`$`" 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
bcftools index A.Assem.sort.pileup.bcf

# Perform the base call per se
bcftools call -v -A -m --ploidy 1 -O v A.Assem.sort.pileup.bcf
-o A.VarBase.vcf
```

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, and 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
 -O v A.Assem.sort.pileup.bcf -o A.AllBase.vcf
```

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_PAO1	1467483	NO	CG/CCGG	NO	CG/CGG	0	0.3	0	0
AE004091.2_PAO1	2239555	AC/AGC	A/AG	A/AG	A/AG	0.9	0.8	0.9	0.9
AE004091.2_PAO1	5156124	A/A	A/C	A/C	A/C	0	0.8	1	1
AE004091.2_PAO1	5307676	NO	NO	AGCC/AGC	NO	0	0	0.8	0
AE004091.2_PAO1	5365634	T/T	T/C	T/C	T/C	0	1	1	1
AE004091.2_PAO1	6006593	NO	NO	NO	G/GTGGCC	0	0	0	0.5

(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 (e.g., 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'
A.Assem.sort.pileup
```

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 Subheading 3.1) or generate clean mutants by reintroducing 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. Restreak the frozen stock for single colonies of mutants carrying a specific mutation (**step 6** in Subheading 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 Subheading 3.1).
4. Analyze antibiotic-related characteristics of individual clones and/or evolved populations as described in Subheading 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* Subheading 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 Subheading 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 100μL of bacteria (**step 3**) with 100μL 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 10μL 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 Subheading 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, 8μg/mL tobramycin). Isolates with varying levels of resistance can then be tested for survival at high antibiotic concentrations as described in Subheading 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 100μL, 500μL, and 1-2 mL of bacteria to obtain spontaneous mutants with resistance levels of 2× MIC, 4× MIC or 8–16× MIC, respectively. Incubate plates overnight. If you do not obtain any spontaneous resistance mutants, repeat this step with more bacteria.
3. Pick ten 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 Subheading 3.1).
5. Measure MIC of the isolated mutants as described in Subheading 3.2.
6. Store a pellet for genome sequence analysis (*see* **step 7** in Subheadings 3.1 and 3.3).
7. Test the survival of each mutant to antibiotic treatment (**steps 3–6** in Subheading 3.5).

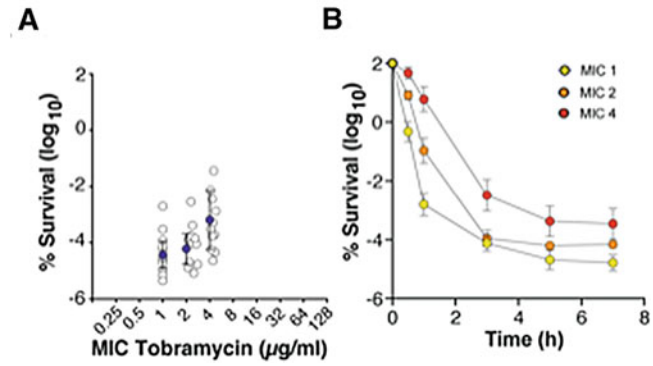

Fig. 2 Resistant variants of the *P. aeruginosa* lab strain PAO1 with different MIC were tested for survival when challenged with 32μg/mL tobramycin. The survival score is plotted as a function of the MIC (**a**) or over time for mutants grouped according to their resistance levels (**b**)

8. Plot survival scores and MIC in the same graph as shown in Fig. 2a or survival scores as a function of time for mutants binned based on MIC values as shown in Fig. 2b.

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 4–5× excess of flasks may be required for the entire experiment.
4. All studies described her used the *P. aeruginosa* PAO1 lab strain as starting point for evolution experiments. All strains should be stored in 15% DMSO-LB at −80 °C. 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 ten 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 10× 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 32μg/mL (32× MIC) and ciprofloxacin at 2.5μg/mL (20× 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 10μg/mL (10× MIC) were used, rapid development of drug resistance was observed (2–3 days). Evolution experiments with drug combinations allowed to reduce tobramycin concentration to 10–16μg/mL, while ciprofloxacin was generally maintained at 2.5μg/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, six 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 10μL of the culture to the agar plate using a multichannel pipette by touching the agar surface with the ends of the six tips followed by gentle ejection. Then slightly tilt the plates and allow the droplets to flow toward 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 overgrown 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 (32μg/mL) or ciprofloxacin (2.5μg/mL) were compared with and without an additional LB wash/resuspension step. While significant differences were observed for nondiluted 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 reengineered 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 sublineages 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 SNP 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 "+" delimitator (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+TTATGC-
GA
CGGTGATGATGGGCGCCGACTACGTCGAGCCGGACCTGGTGATGACCCGCGACGG-
CAAGCTGGTCG
+
BBBBBEFGGGGGGGGGGGDGGGGGGGGGGFGGGGGGGGGGGEGGGGGGGGGEECGG
@DGGGGGGGG
```

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 miss-interpreted 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 art factual variants will appear constant and will therefore easily be ruled out.

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References

1. Hajjeh R, Mafi A (2016) The growing threat of antibiotic resistance in the Eastern Mediterranean region—what does it take to control it? East Mediterr Heal J 22:701–702
2. Andersson DI, Balaban NQ, Baquero F et al (2020) Antibiotic resistance: turning evolutionary principles into clinical reality. FEMS Microbiol Rev 44:171–188
3. Horne D, Tomasz A (1977) Tolerant response of *Streptococcus sanguis* to beta-lactams and other cell wall inhibitors. Antimicrob Agents Chemother 11:888–896

4. Brauner A, Fridman O, Gefen O et al (2016) Distinguishing between resistance, tolerance and persistence to antibiotic treatment. Nat Rev Microbiol 14:320–330
5. Hegreness M, Shoresh N, Damian D et al (2008) Accelerated evolution of resistance in multidrug environments. Proc Natl Acad Sci U S A 105:13977–13981
6. Toprak E, Veres A, Michel J-B et al (2011) Evolutionary paths to antibiotic resistance under dynamically sustained drug selection. Nat Genet 44:101–105
7. Munck C, Gumpert HK, Wallin AIN et al (2014) Prediction of resistance development against drug combinations by collateral responses to component drugs. Sci Transl Med 6:262ra156
8. Rodriguez De Evgrafov M, Gumpert H, Munck C et al (2015) Collateral resistance and sensitivity modulate evolution of high-level resistance to drug combination treatment in *Staphylococcus aureus*. Mol Biol Evol 32:1175–1185
9. Lázár V, Nagy I, Spohn R et al (2014) Genome-wide analysis captures the determinants of the antibiotic cross-resistance interaction network. Nat Commun 5:4352
10. Fridman O, Goldberg A, Ronin I et al (2014) Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. Nature 513:418–421
11. Van den Bergh B, Michiels JE, Wenseleers T et al (2016) Frequency of antibiotic application drives rapid evolutionary adaptation of *Escherichia coli* persistence. Nat Microbiol 1:16020
12. Khare A, Tavazoie S (2020) Extreme antibiotic persistence via heterogeneity-generating mutations targeting translation. mSystems 5:1–12
13. Sulaiman JE, Lam H (2020) Proteomic investigation of tolerant *Escherichia coli* populations from cyclic antibiotic treatment. J Proteome Res 19:900–913
14. Mechler L, Herbig A, Paprotka K et al (2015) A novel point mutation promotes growth phase-dependent daptomycin tolerance in *Staphylococcus aureus*. Antimicrob Agents Chemother 59:5366–5376
15. Michiels JE, Van den Bergh B, Verstraeten N et al (2016) In vitro emergence of high persistence upon periodic aminoglycoside challenge in the ESKAPE pathogens. Antimicrob Agents Chemother 60:4630–4637
16. Levin-Reisman I, Ronin I, Gefen O et al (2017) Antibiotic tolerance facilitates the evolution of resistance. Science 355:826–830
17. Windels EM, Michiels JE, Fauvart M et al (2019) Bacterial persistence promotes the evolution of antibiotic resistance by increasing survival and mutation rates. ISME J 13:1239–1251
18. Liu J, Gefen O, Ronin I et al (2020) Effect of tolerance on the evolution of antibiotic resistance under drug combinations. Science 367:200–204
19. den Bergh V, Michiels JE, Michiels J (2016) Experimental evolution of *Escherichia coli* persister levels using cyclic antibiotic treatments. In: Michiels J, Fauvart M (eds) Bacterial persistence: methods and protocols. Springer, New York, NY, pp 131–143
20. Santi I, Manfredi P, Maffei E, Egli A, Jenal U (2021) Evolution of antibiotic tolerance shapes resistance development in chronic *Pseudomonas aeruginosa* infections. mBio 12(1): e03482-20. https://doi.org/10.1101/2020.10.23.352104
21. Wiegand I, Hilpert K, Hancock REW (2008) Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 3:163–175
22. Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. Nat Methods 9:357–359
23. Li H (2011) A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. Bioinformatics 27:2987–2993
24. Wall L, Christiansen T, Orwant J (2000) Programming perl, 3rd edn. O'Reilly Media, Sebastopol

Part V

Persister Cell Resuscitation and Eradication

Chapter 13

Detecting Persister Awakening Determinants

Dorien Wilmaerts, Jan Michiels, and Natalie Verstraeten

Abstract

For long, persistence research has focused primarily on disentangling mechanisms of persister state entry. Due to the rapid advances in the field of single-cell techniques and newly obtained insights in the persister phenotype, studying persister awakening has been unlocked and it has gained much interest in the scientific community. However, a framework on how this research should be conducted is currently lacking. Therefore, we here present a method to detect and validate genes important for persister awakening.

Key words Persister, Persister awakening, Single-cell, Cell sorting

1 Introduction

Recent work has brought to light increasing evidence on the role of persister cells in the recurrent nature of many chronic infections. In addition, in vitro lab work has established that persisters form a stepping stone for resistant mutants to emerge and it will only be a matter of time before the same evidence is unveiled in vivo [1–3]. Furthermore, periodic antibiotic treatment, resembling the current clinical practice, was shown to select for a high-persistence phenotype [4–6]. Hence, the burden of persisters residing in bacterial populations is a growing public health concern worldwide. The number of persisters in a given population is determined by the rates of persister formation and awakening [7]. As a consequence, an increased or decreased persister fraction can be attributed to changes in formation and/or awakening (Fig. 1). Only few studies have investigated mechanisms underlying persister awakening, mainly because studying the latter requires dedicated techniques to observe changes at the single-cell level. As a result, differences in

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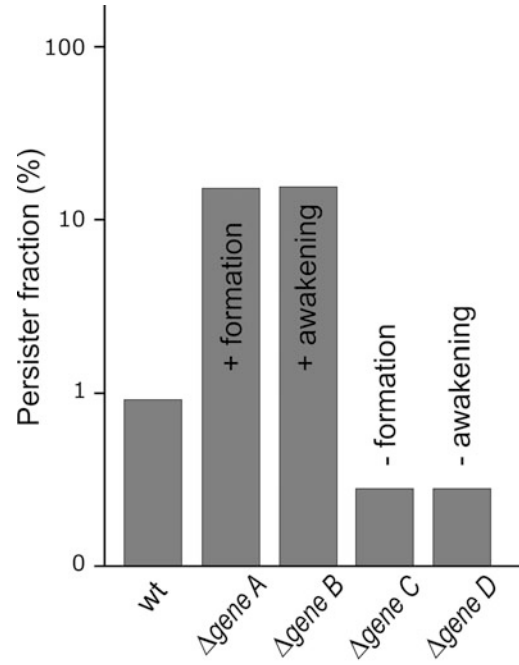

Fig. 1 When quantifying persister levels by counting colony-forming units (CFUs) following antibiotic treatment, an increase in the formation and/or the awakening of persister cells results in an increased persister fraction. Similarly, a decrease in the formation and/or the awakening of persister cells results in a decreased persister fraction

persister fractions are often assigned to changes in the formation of persister cells, ignoring possible changes in persister awakening kinetics.

A limited number of mechanisms have been described to contribute to persister awakening. First, persisters have been shown to accumulate DNA damage during fluoroquinolone treatment, and the repair of this damage has been demonstrated to be a critical step for persister survival [8, 9]. Second, toxin-induced persisters have been found to rely on deactivation of the toxin for their awakening. In *Salmonella*, Pth presumably deactivates TacT-induced persisters [10]. HokB-persisters rely on DsbC-mediated monomerization of HokB peptides, making them susceptible to degradation by the DegQ protease. Removal of the pore-forming HokB toxin allows the electron transport chain to repolarize the membrane [11]. Third, while the formation of aggregates has been associated with the induction of persistence, the removal of these aggregates has been associated with persister awakening [12, 13]. Although detailed molecular mechanisms are currently unknown, the protease DnaK has been shown to be vital for disaggregation of the aggregates [12].

A better understanding of the molecular mechanisms underlying persister awakening might prove useful for combatting chronic infections and preventing the emergence of resistant mutants, as waking up renders a persister cell susceptible to antibiotics [14]. In this chapter, we present a procedure to identify genes important for persister awakening or mutants with altered awakening kinetics upon dilution in fresh medium, and we give suggestions on how to validate the role of these genes in persister awakening.

2 Materials

Prepare all media using deionized water. Store at room temperature, unless indicated otherwise. *See* also **Note 1**.

1. Lysogeny broth (LB): For 1 L, use 10 g NaCl, 10 g tryptone, and 5 g yeast extract. Autoclave the solution prior to use.
2. LB agar plates: Prepare LB medium as above. Add 15 g agar per L LB medium. Autoclave the solution and store at 50 °C. Poured plates should be stored at 4 °C in closed bags with the agar side up for maximum 2 weeks.
3. 10 mM magnesium sulfate solution: 2.46 g of $MgSO_4.7H_2O$ in 1 L of distilled water. Autoclave prior to use.
4. Phosphate buffer saline solution (PBS, 1×): For 1 L, add 0.2 g KCl, 0.24 g KH_2PO_4, 8 g NaCl, and 1.15 g Na_2HPO_4. Adjust the pH to 7.4. Autoclave the solution.
5. Antibiotic stock solution (100×): For ofloxacin, prepare a stock solution of 0.5 mg/mL (*see* **Note 2**). For tobramycin, prepare a stock solution of 40 mg/mL. Store the solutions at −20 °C.
6. Dye: Baclight™ RedoxSensor™ Green Vitality Kit (store at −20 °C). This kit provides redox sensor green staining (RSG), propidium iodide (PI), sodium azide, and carbonyl cyanide m-chlorophenyl hydrazine (CCCP).
7. BD Influx™ cell sorter or equivalent.
8. CytoFLEX S flow cytometer with a plate loader module or equivalent.
9. Sterile 96-well plates, flat-bottom.
10. Sterile Eppendorf tubes (1.5 mL).
11. Sterile glass test tubes (25 mL).
12. Sterile plastic tubes for sorting.
13. Nikon Eclipse TI-E inverted microscope equipped with an incubation chamber or equivalent.
14. Time-lapse microscopy equipment: microscopy slides, coverslips, 25 μL gene frames.

3 Methods

Deletion of a plethora of genes and multiple genetic mutations alter the persister fraction [15]. In this chapter we describe two protocols that can be used to determine whether mutation of a gene of interest affects the formation or the awakening of persister cells in a given time frame. Both protocols make use of the redox sensor green (RSG) dye, which emits green light when cell are metabolically active. This metabolic activity is correlated with the reducing capacity of the cell. Identifying genes important for persister awakening is a first step toward unraveling molecular mechanisms underlying persister awakening.

Unless noted otherwise, all incubations of liquid samples are performed at 37 °C under shaking conditions (200 rpm). Plates are incubated at 37 °C in static conditions, with the agar side up. *See* also **Note 3**.

3.1 Identification of Persister Awakening Genes by FACS

By staining cells after antibiotic treatment using RSG, metabolically active RSG+ cells can be sorted using fluorescence-activated cell sorting. By plating out and counting the number of colonies, the fraction of regrowing persister cells over metabolically active cells can be determined [11]. Comparing results for wild-type and mutant strains allows to quantify the effect of specific mutations on persister awakening.

3.1.1 Persister Assay and Control Samples

1. Start an overnight culture by inoculating a single colony from a plate in a test tube containing 5 mL LB and incubate for 24 h.
2. After incubation, dilute the overnight culture 100× in 5 mL LB and incubate again for 16 h.
3. Treat 990 μL of the culture with 10 μL antibiotics (ofloxacin or tobramycin) in a sterile, glass test tube. As a control, treat 990 μL of the culture with 10 μL sterile, deionized water in a sterile, glass test tube. Incubate the samples for 5 h.
4. Prepare an exponentially growing culture to be used as a positive control. To this end, dilute an overnight-grown culture 100× in 5 mL fresh LB and incubate for 3 h.
5. Keep an overnight-grown culture in the incubator, as it will be used to prepare the negative controls.

3.1.2 Preparatory Work

Approximately 1 h before the end of the antibiotic treatment period, start preparing the experiment.

1. Add 1 mL PBS to labeled plastic tubes in which the cells will be sorted.
2. Prepare the agar plates and Eppendorf tubes and add 180 μL of 10 mM $MgSO_4$ to the wells of a 96-well plate which will be

used for making dilutions. Leave blank wells for including the samples.

3. Allow RSG to warm to room temperature. For gram-negative bacteria, dilute RSG 1000× in PBS, which should also be at room temperature (*see* **Note 4**). Prepare 1 mL of this solution per sample, taking into account the positive control culture (i.e., an exponentially growing culture displaying high metabolic activity), and the two negative control cultures [i.e., stationary-phase cultures with decreased RSG values (*see* **Note 5**)]. Add 1 mL of PBS + RSG in prelabeled tubes compatible with the used cell-sorting device.

3.1.3 Sample Preparation

1. Allow the negative control reagents sodium azide and CCCP, included in the BacLight redox sensor kit, to warm to room temperature. Per mL of overnight-grown culture, add either 5 μL sodium azide or 2 μL CCCP (*see* **Note 5**). Allow the reagents to react with the cells (1–5 min) and subsequently prepare the negative control samples by diluting the cells 100× in a tube with 1 mL PBS + RSG.
2. Dilute the exponentially growing culture (positive control) 50× in a tube with 1 mL PBS + RSG.
3. Dilute the untreated samples 100× in 1 mL PBS + RSG.
4. Centrifuge (5 min, 4000 × *g*) 500 μL of the antibiotic-treated samples and resuspend in 1 mL PBS + RSG.
5. Incubate all samples for 15 min at room temperature.

3.1.4 Sorting and Plating Metabolically Active Cells

1. Measure the green fluorescence of the exponentially growing culture (positive control) (*see* **Note 6**). Choose an appropriate gate (Population 1, P1) that encompasses all RSG+ cells.
2. Measure the green fluorescence of the negative control cultures. Adjust the borders so the P1 gate does not encompass RSG− cells (Fig. 2). This gate can subsequently be used to sort metabolically active cells.
3. For each untreated sample, sort 10,000 RSG+ cells from P1 in 1 mL PBS. Vortex the tubes and add 200 μL of each sample to the empty wells of a 96-well plate (*see* **step 2** in Subheading 3.1.2). Dilute until 10^{-2} and plate out 10^{0}, 10^{-1}, 10^{-2}.
4. For each antibiotic-treated sample, sort 2000 cells from P1 in 1 mL PBS (*see* **Notes 7** and **8**). Vortex the tubes and transfer the content to an Eppendorf tube. Centrifuge the Eppendorf tubes for 5 min at 4000 × *g* (*see* **Note 9**). Carefully remove the supernatant and resuspend the cells in 110 μL of 10 mM $MgSO_4$. Directly plate out 100 μL and add 90 μL of 10 mM $MgSO_4$ to the remaining 10 μL in the Eppendorf tube. Plate out the resulting 100 μL.
5. Incubate the plates 48 h prior to counting.

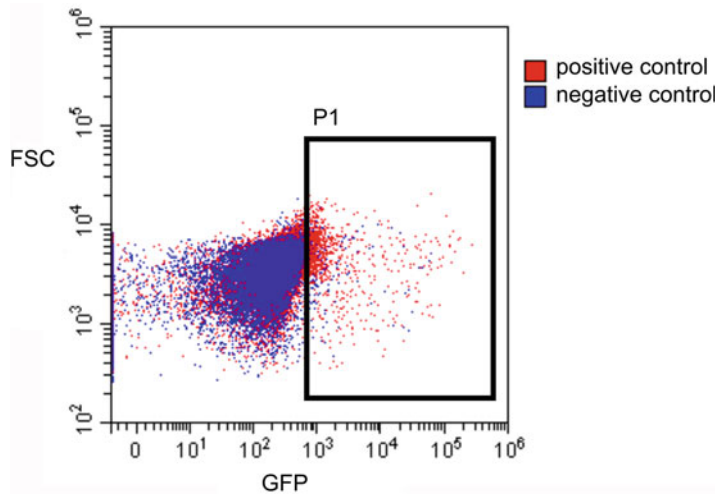

Fig. 2 Example of a P1 gate encompassing RSG+ cells. The negative control is indicated in blue, the positive control is indicated in red. *FSC* forward scatter

3.1.5 Data Analysis

1. For each strain under investigation, count the number of colonies on the plates. Determine the number of growing cells (on plate) per sorted metabolically active cell, both for the treated sample and the untreated sample. Divide the number of colony-forming units (CFUs) per metabolically active cell in the treated sample over the number of CFUs per metabolically active cell in the untreated sample. This way, small differences in cell survival after sorting that can arise from differences in genotype are taken into account.
2. Calculate whether there is a statistical difference in number of persister cells per metabolically active cell between the mutants and the wild type. If the fraction is lower for a mutant, this means that persister awakening is impeded. If the fraction is higher, persister awakening is enhanced. Protocols described in Subheading 3.3 can be used for further validation.

3.2 Identification of Persister Awakening Genes by Flow Cytometry

If a cell-sorting device is not available, the abovementioned protocol can be adapted slightly. Instead of sorting and plating out metabolically active cells, a persister assay is performed in parallel with the quantification of the metabolically active cells in the sample.

3.2.1 Persister Assay and Control Samples

1. Start an overnight culture by inoculating a single colony from a plate in a test tube containing 5 mL LB and incubate for 24 h.
2. After incubation, dilute the overnight culture 100× in 5 mL LB and incubate again for 16 h.
3. Treat 990 μL of the culture with 10 μL antibiotics (ofloxacin or tobramycin) in a sterile, glass test tube. As a control, treat

990 μL of the culture with 10 μL sterile, deionized water. Incubate the samples for 5 h.

4. Prepare an exponentially growing culture to be used as the positive control. To this end, dilute an overnight-grown culture 100× in 5 mL fresh LB and incubate for 3 h.
5. Keep an overnight-grown culture in the incubator, as this will be used to prepare the negative controls.

3.2.2 Preparatory Work

Approximately 1 h before the end of the antibiotic treatment period, start preparing the experiment.

1. Prepare the agar plates and add 180 μL of 10 mM $MgSO_4$ to the wells of a 96-well plate which will be used for making the tenfold dilution series. Leave blank wells for including the samples.
2. Allow RSG to warm to room temperature. For gram-negative bacteria, dilute RSG 1000× in PBS, which should also be at room temperature (*see* **Note 4**). Prepare 200 μL of this solution per sample. Prepare a 96-well plate which will be used in the cytometer by filling the wells with 198 μL PBS + RSG, taking into account the positive control culture (i.e., an exponentially growing culture displaying high metabolic activity), and the two negative control cultures [i.e., stationary-phase cultures with decreased RSG values (*see* **Note 5**)].

3.2.3 Sample Preparation

1. Dilute the exponentially growing culture (positive control) 10× in 10 mM $MgSO_4$. Add 2 μL of the diluted sample to a well containing 198 μL PBS + RSG.
2. Prepare the negative controls. Allow the negative control reagents sodium azide and CCCP, included in the BacLight redox sensor kit, to warm to room temperature. Dilute an overnight-grown culture 10× in 10 mM $MgSO_4$. Per 100 μL of sample, add 0.5 μL sodium azide or 0.2 μL CCCP (*see* **Note 5**). Allow the reagents to react with the cells (1–5 min). Subsequently, add 2 μL of the negative controls to two wells containing 198 μL PBS + RSG.
3. Add 200 μL of the untreated and treated samples to the blank wells of a 96-well plate filled with 10 mM $MgSO_4$ (*see* **step 1** in Subheading 3.2.2). Dilute the untreated sample 10×. Transfer all samples to a 96-well plate containing PBS + RSG. Upon dilution of the samples to PBS + RSG, make sure that there are between 10^5 and 10^6 cells in each well. As a rule of thumb, for untreated samples add 2 μL of the 10^{-1} dilution to 198 μL PBS + RSG. For treated samples add 2 μL of the 10^0 dilution to 198 μL PBS + RSG.
4. Incubate all samples for 15 min in a dark environment at room temperature.

3.2.4 Measuring the Number of Metabolically Active Cells

1. To select metabolically active cells, measure the green fluorescence of the exponentially growing culture (positive control) (*see* **Note 6**). Choose an appropriate gate (P1) that encompass all RSG+ cells.
2. Measure the green fluorescence of the negative control cultures. Check whether the P1 gate does not encompass RSG− cells.
3. Run the positive and negative control wells. Record 10,000 cells.
4. Measure RSG fluorescence in antibiotic-treated and untreated samples. Record 10,000 cells per well. Make sure that the number of events per μL is recorded.

3.2.5 Plating Out the Samples

1. While the flow cytometer is running, take the previously filled 96-well plate with 10 mM $MgSO_4$ and 200 μL of the samples. Dilute and plate out the appropriate dilution of all samples and controls which will give countable plates (*see* **Note 10**).
2. Incubate the plates for 48 h prior to counting.

3.2.6 Data Analysis

1. Count the plates and determine the number of CFUs per mL.
2. Reevaluate the P1 gate using the positive and negative control samples and adjust if necessary. Determine the number of events per μL in P1 for every sample. Take into account the dilution factor to determine the number of metabolically active cells per mL.
3. Determine the number of cells (grown on plate) over the number of metabolically active cells measured in the sample and take the log. Ideally, the control samples should be close to zero.
4. Calculate whether there is a statistical difference in number of persister cells per metabolically active cell between the mutants and the wild type. If the fraction is lower for a mutant, this means that persister awakening is impeded. If the fraction is higher, persister awakening is enhanced. Protocols described in Subheading 3.3 can be used for further validation.

3.3 Validation by Single-Cell Growth Dynamics

The above-described protocols yield detailed information on the fraction of regrowing persister cells over the metabolically active cells after antibiotic treatment of a population. There are several additional experiments that can be performed to further validate the importance of a specific gene in persister awakening. For example, in case a knock-out mutant was assessed, this deletion can be complemented. In a first step, complementation can be performed before antibiotic treatment. In a next step, it might be interesting to try to complement the gene at other time points, for example

after treatment. It is important to note that complementation after treatment might not work, as the persister cells need to produce the protein of interest while in the persister state.

If a gene is important for persister awakening, deletion impedes the awakening of a large fraction of the cells. In addition, it is highly likely that awakening kinetics of these mutants are affected. Therefore, validation of the timing of awakening and the timing of cell division might prove useful.

3.3.1 Sample Preparation

1. Inoculate a single colony from an agar plate in a test tube containing 5 mL LB medium and incubate for 24 h.
2. Dilute the preculture 100× in 5 mL LB medium and incubate for 16 h.
3. Treat 990 μL of the culture with 10 μL of the stock solution of ofloxacin or tobramycin.
4. Incubate for 5 h.
5. Prepare an LB agar pad. Make 100 mL LB medium and add 2 g agarose. Heat in the microwave until the agarose is dissolved. Attach a gene frame to a microscope glass slide. Add 500 μL LB agar in the frame and gently press a cover slip on the frame to flatten the agar. Keep the cover slip on the agar and store at room temperature until use.
6. Centrifuge 500 μL of the sample (5 min, 4000 × *g*).
7. Resuspend the pellet in an equal volume of preheated LB medium (37 °C).
8. Repeat the wash step.
9. Add 2 μL of the sample to the LB agar pad. Spread the drop by tapping the glass slide. Wait until it is dry before covering the pad with a glass cover slip.

3.3.2 Visualization of Regrowth Using Time-Lapse Microscopy

1. Choose a sufficient number of frames that will be followed in time (*see* **Note 11**). The frames should not contain too many cells, as this prevents following the cells for a sufficient period of time (*see* **Note 12**).
2. Visualize the cells every 15 min for at least 20 h. This way, slowly awakening mutants can be monitored.

3.3.3 Data Analysis

1. A persister cell is defined as a cell that is able to provide a viable progeny. Therefore, monitor the cells that are able to divide multiple times. Dividing only once is not sufficient to be termed a persister cell.
2. Follow the cell length over time for the persister cells.
3. For every persister cell, characterize the timing of awakening and the timing of cell division. This timing of awakening is the time point when the cell reaches, for example, twofold its initial

size. Particularly in situations where persister cells elongate strongly before cell division, as demonstrated after fluoroquinolone treatment [8, 16], this timing of awakening is a useful parameter to compare between different strains.

4. Compare the timing of awakening and the timing of cell division between the awakening mutants and the wild type.
5. Make a frequency distribution of the awakening times. While a normal distribution indicates that several molecular mechanisms underlie persister awakening, an exponential distribution indicates that awakening is stochastically determined [17].

4 Notes

1. The protocols described in this chapter are validated for *Escherichia coli* BW25113 and TOP10 in LB medium combined with ofloxacin or tobramycin treatment. Optimization of the protocols is advisable when using other species, strains, media or antibiotics.
2. Add drops of 1 M HCl to increase solubility of ofloxacin in deionized water.
3. The described protocols are based on a persister assay and are used to investigate stationary-phase persisters in *E. coli*. However, the used time schedule can be modified to study for example exponential-phase persisters.
4. When gram-negative bacteria are used, RSG should be diluted 10,000×. Check the manufacturer's specifications before use.
5. The kit provides two methods to decrease the RSG value of the sample, based on either sodium azide or CCCP. In a first run, check which of the two methods works best and only include one of both in subsequent runs.
6. RSG fluorescence is measured using a 488 nm laser and a 520 nm filter.
7. Optimization of the number of sorted cells in antibiotic-treated samples might be necessary. Normally, 1500–2000 cells are sorted. However, in case samples have many RSG+ cells, the number of sorted cells can be increased. The time needed for sorting should however be limited as storing the sorted samples in PBS for prolonged periods of time affects cell survival.
8. After sorting, plate out the samples as soon as possible to increase survival.
9. Remember the orientation of the tubes in the centrifuge as a pellet will not be visible.

10. When plating out samples that are only diluted 10× or 100×, make sure the antibiotic is removed from the sample. To this end, centrifuge the sample for 5 min at 4000 × *g*. Remove the supernatant and add an equal volume of 10 mM $MgSO_4$. Repeat this twice before making the dilution series.
11. For a persister fraction of approximately 1%, 30 frames are sufficient for observing regrowing persister cells. If the persister fraction is lower, more frames are needed. However, optimization might be necessary.
12. If the cell density is too high, dilute the sample 2×.

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References

1. Sebastian J, Swaminath S, Nair RR et al (2016) *De novo* emergence of genetically resistant mutants of *Mycobacterium tuberculosis* from the persistence phase cells formed against antituberculosis drugs *in vitro*. Antimicrob Agents Chemother 61:e01343–e01316
2. Windels EM, Michiels JE, Fauvart M et al (2019) Bacterial persistence promotes the evolution of antibiotic resistance by increasing survival and mutation rates. ISME J 13:1239–1251
3. Levin-Reisman I, Ronin I, Gefen O et al (2017) Antibiotic tolerance facilitates the evolution of resistance. Science 355:826–830
4. Michiels JE, Van den Bergh B, Verstraeten N et al (2016) *In vitro* emergence of high persistence upon periodic aminoglycoside challenge in the ESKAPE pathogens. Antimicrob Agents Chemother 60:4630–4637
5. Mechler L, Herbig A, Paprotka K et al (2015) A novel point mutation promotes growth phase-dependent daptomycin tolerance in *Staphylococcus aureus*. Antimicrob Agents Chemother 59:5366–5376
6. Van den Bergh B, Michiels JE, Wenseleers T et al (2016) Frequency of antibiotic application drives rapid evolutionary adaptation of *Escherichia coli* persistence. Nat Microbiol 1:16020
7. Michiels JE, Van den Bergh B, Verstraeten N et al (2016) Molecular mechanisms and clinical implications of bacterial persistence. Drug Resist Updat 29:76–89
8. Völzing KG, Brynildsen MP (2015) Stationary-phase persisters to ofloxacin sustain DNA damage and require repair systems only during recovery. mBio 6:e00731–e00746
9. Mok WWK, Brynildsen MP (2018) Timing of DNA damage responses impacts persistence to fluoroquinolones. Proc Natl Acad Sci U S A 115:e6301–e6309
10. Cheverton AM, Gollan B, Przydacz M et al (2016) A *Salmonella* toxin promotes persister formation through acetylation of tRNA. Mol Cell 63:86–96
11. Wilmaerts D, Dewachter L, De Loose P-J et al (2019) HokB monomerization and membrane repolarization control persister awakening. Mol Cell 75:1031–1042
12. Pu Y, Li Y, Jin X et al (2019) ATP-dependent dynamic protein aggregation regulates bacterial dormancy depth critical for antibiotic tolerance. Mol Cell 73:143–156
13. Yu J, Liu Y, Yin H et al (2019) Regrowth-delay body as a bacterial subcellular structure marking multidrug-tolerant persisters. Cell Discov 5:8

14. Fauvart M, de Groote VN, Michiels J (2011) Role of persister cells in chronic infections: clinical relevance and perspectives on anti-persister therapies. J Med Microbiol 60:699–709
15. Wilmaerts D, Herpels P, Michiels J, Verstraeten N (2019) Genetic determinants of persistence in *E. coli*. In: Lewis K (ed) Persister cells and infectious disease. Springer
16. Goormaghtigh F, Van Melderen L (2019) Single-cell imaging and characterization of *Escherichia coli* persister cells to ofloxacin in exponential cultures. Sci Adv 5:eaav9462
17. Norman TM, Lord ND, Paulsson J et al (2015) Stochastic switching of cell fate in microbes. Annu Rev Microbiol 69:381–403

Chapter 14

Monitoring Persister Resuscitation with Flow Cytometry

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Abstract

Persister cells are defined as a small fraction of phenotypic variants in a cell population that are temporarily tolerant to bactericidal antibiotics. Persisters are not mutant cells; they generally survive lethal concentrations of antibiotics due to their transient nongrowing state. Persister cells have the ability to resuscitate after the end of antibiotic treatment. Despite significant advancements in the understanding of the molecular mechanisms underlying persister formation, we still have little information about their resuscitation mechanisms. In this chapter, we describe a method to detect and monitor persister resuscitation at the single-cell level using flow cytometry analysis. This method enables us to not only assess the resuscitation characteristics of persisters but also determine and quantify various subpopulations in antibiotic-treated cultures, including viable but nonculturable (VBNC) and dead cells.

Key words *Escherichia coli*, Persister resuscitation, Flow cytometry, Viable but nonculturable cells, Beta lactams, Protein dilution, Antibiotic tolerance

1 Introduction

Persistence is a nongenetic, nonheritable survival mechanism that allows organisms to withstand undesirable environmental conditions [1–6]. Persister cells have been identified in many prokaryotic and eukaryotic cell types [3]. They can form stochastically due to random fluctuations in levels of stress-related cellular molecules [3, 6], including those involved in the DNA-damage response [7, 8], ppGpp-dependent stringent response [9–11], toxin–antitoxin systems [12–15], and reactive oxygen species (ROS) [16–18]. Environmental factors, such as antibiotic treatment [8, 19], nutrient depletion [10, 11, 20], stationary-phase respiration [21–23], and osmotic/pH conditions [24, 25], can also affect the level of persister cells present in a cell population. Both stochastic and deterministic mechanisms underlying persister cell formation are currently under intense investigation.

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Persister cells in a growing cell population (e.g., exponential-phase culture) are very rare [4, 26]. These cells are genetically identical to their antibiotic-sensitive kin [3]. When persister cells are recultured in fresh medium after the removal of antibiotic, they can exit from the persistence state (i.e., resuscitate) [2, 4], proliferate like normal cells [4, 27], and form antibiotic-sensitive cell populations [3, 5]. Resuscitation is a hallmark of the persistence phenotype, which underlies the fundamental basis for the clonogenic survival assays [2, 26], enabling us to quantify persister cells in a cell population. Although the resuscitation mechanisms of ofloxacin-treated stationary-phase cells [7, 28], chemically induced dormant cells [29, 30], and various antibiotic-tolerant cell populations generated by the overexpression of toxins and ppGpp molecules [31, 32] have been reported, many resuscitation aspects of native (i.e., noninduced) persister cells still remain to be investigated.

Microscopy has been extensively used to monitor native persister cells during and/or after antibiotic treatments [4, 33, 34]. However, with microscopy, only a limited number of persister cells can be monitored; whether these reported cells represent the entire persister subpopulation needs to be validated. Although persister cells have been successfully investigated with microfluidic devices [4, 33] and flow cytometry [27, 35, 36], their low abundance, transient state, and similarities to VBNC phenotypes still represent significant challenges in the field [27, 37–39]. Here, we describe our published methodology [36] where we use flow cytometry analysis, beta-lactam-mediated cell lysis, cell division and clonogenic survival assays [27, 40] to monitor persister cell resuscitation.

2 Materials

Prepare all growth media and solutions using deionized (DI) water (18.2 MΩ-cm at 25 °C) and store at room temperature unless stated otherwise. Use sterile conditions and instruments when required. Work either in a biological safety cabinet or next to a Bunsen burner flame on a bench. Use 70% (v/v) ethanol solution whenever needed to sterilize the working area. Attentively maintain all safety protocols while performing the experiments. Cells are grown at 37 °C in all conditions.

1. Frozen cells stock: Streak the desired bacterial strain on an agar plate. After growing the agar plate in an incubator for 16 h, pick a single colony from the plate and inoculate in 2 mL of LB broth in a 14-mL test tube and grow the culture in a shaker at 250 revolutions per min (rpm). When the cells reach the

stationary phase, mix 500 μL of cell culture with 500 μL 50% sterile glycerol in a cryogenic vial and store the vial at −80 °C.

2. Bacterial strain: Use a bacterial strain harboring a fluorescent protein expression system. We used an *E. coli* MG1655 strain that has an inducible mCherry expression system (*see* **Note 1**).
3. Luria-Bertani (LB) broth: Dissolve 5 g yeast extract, 10 g tryptone, and 10 g NaCl in 1 L DI water, and then autoclave the liquid medium (*see* **Note 2**). Store the medium in a dark place at room temperature. Do not use LB broth that is older than 4 weeks [41].
4. LB agar: Add 5 g yeast extract, 10 g tryptone, 10 g NaCl, and 15 g agar to 1 L DI water. Sterilize the medium by autoclaving (*see* **Note 2**). After cooling down the medium on the working bench (~50–60 °C), pour the medium in petri dishes (~25 mL/square plate). Leave the plates on the bench overnight to dry properly. Store them at 4 °C.
5. 1× phosphate buffered saline (PBS) solution: Add 100 mL of a sterile 10× PBS solution to 900 mL of sterile DI water and mix. The resulting 1× PBS (pH 7.4 ± 0.1) solution contains 11.9 mM phosphates (sodium phosphate dibasic and potassium phosphate monobasic), 137 mM sodium chloride, and 2.7 mM potassium chloride. Store the buffer solution at room temperature for future use (*see* **Note 3**).
6. Antibiotic stock solution: Weigh 100 mg of beta-lactam powder (i.e., ampicillin sodium salt) and mix with 1 mL of DI water by vortexing. Sterilize the resulting solution using a membrane filter (0.2μm filter). Prepare aliquots (~50 μL/microcentrifuge tube) and store them at −20 °C (*see* **Note 4**).
7. 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) solution: Weigh 238.31 mg of IPTG and add 1 mL of DI water. Make the solution sterile using a membrane filter. Prepare the aliquots (~50 μL/microcentrifuge tube) and store them at −20 °C (*see* **Note 4**).
8. 70% ethanol (v/v) solution: Add 300 mL of DI water to 700 mL of denatured ethanol (200 proof). Alternatively, purchase 70% denatured ethanol.
9. Sterile polypropylene and polystyrene plastic test tubes (5-mL, 14-mL, 50-mL). We used 5-mL round-bottom (snap capped) tubes for flow cytometry experiments and 14-mL round bottom (snap capped) test tubes for overnight precultures. For centrifugation purposes, we used 50-mL conical tubes (screw capped).
10. Sterile 250-mL Erlenmeyer (baffled) flasks (*see* **Note 5**).
11. Sterile 1.8-mL or 2-mL microcentrifuge tubes.

12. Sterile square petri dishes to prepare agar plates for quantitation of colony formation units (CFU).
13. Sterile pipette tips with various volume capacities (*see* **Note 6**).
14. Sterile serological pipettes (25-mL).
15. Benchtop centrifuge for 50-mL conical tubes.
16. Benchtop centrifuge for microcentrifuge tubes.
17. A shaker with test-tube and flask holders. The shaker should be temperature and rotation speed controlled.
18. Temperature-controlled incubators for agar plates.
19. Sterile 96-well round-bottom and flat-bottom plates.
20. A plate reader to measure the optical density at 600 nm (OD_{600}) wavelength in a 96-well format (*see* **Note 7**).
21. Sterile 50-mL chemical reservoirs.
22. A flow cytometer (*see* **Note 8**).
23. Quality control (QC) particles (*see* **Note 9**).

3 Methods

We used an IPTG-inducible mCherry expression system [21, 22, 38, 39] to monitor persister resuscitation with flow cytometry. IPTG is added in cell cultures to induce mCherry expression to maintain a high red fluorescence signal in cells (Fig. 1a). Cells are treated with ampicillin at exponential phase to lyse the antibiotic-sensitive cells. Nonlysed cells, containing persister and VBNC cells [27, 39], retain high fluorescence (Fig. 1a) [36]. After antibiotic treatment, cells are washed to remove the chemicals and transferred to fresh medium. Persister cells, unlike VBNC cells, have the ability to resume proliferation upon the removal of antibiotics [27, 37–39]. In the absence of inducer, flow cytometry reports continuing cell division of resuscitated cells as evidenced by dilution of the mCherry protein (Fig. 1b) [36]. Side (SSC) or forward scattered (FSC) signals of growing cells are expected to increase (Fig. 1b) [27, 35, 42, 43]. We performed all flow cytometry experiments at room temperature. The samples were prepared by suspending ~10^6 to 10^7 cells in 1 mL of PBS [22].

3.1 Preparing Overnight Precultures

1. Inoculate cells harboring an IPTG-inducible mCherry expression system from a frozen stock in 2 mL sterile LB medium in a 14-mL round bottom tube with the inducer (1 mM IPTG) and culture in a shaker at 250 rpm for 24 h.
2. Similarly, inoculate wild-type cells (mCherry-negative control) from a frozen cell stock in fresh LB medium with 1 mM IPTG and culture for 24 h.

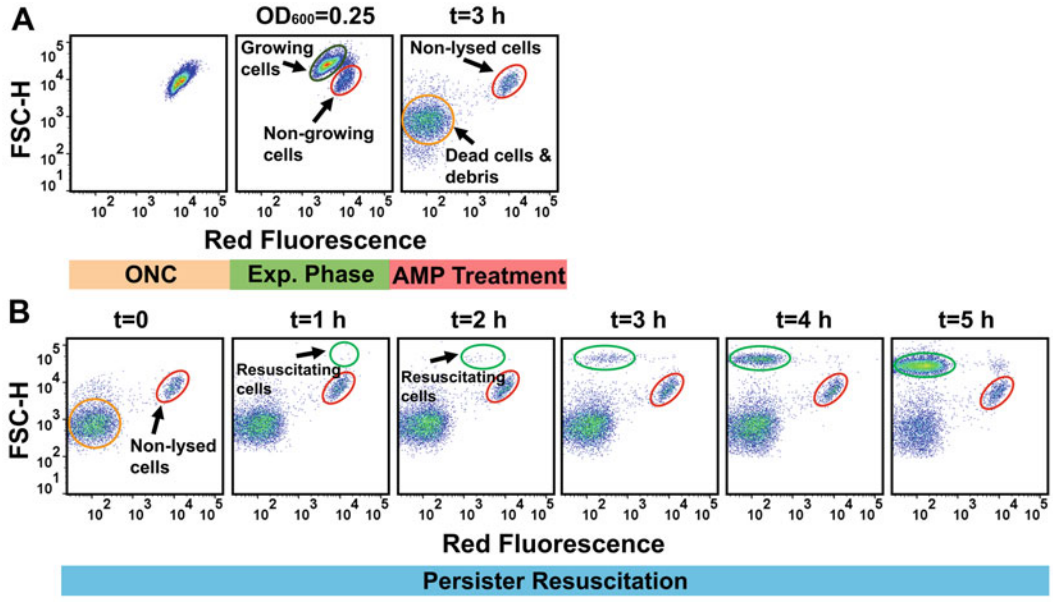

Fig. 1 Beta-lactam mediated cell lysis and persister resuscitation assays. (**a**) mCherry-positive stationary-phase cells were diluted 100-fold in 25 mL of LB medium containing 1 mM IPTG. When the culture reached the exponential phase ($OD_{600} = 0.25$), the cells were treated with ampicillin at 600μg/mL (100× MIC). Samples from the culture were collected at indicated time points to monitor lysed and nonlysed cells with a flow cytometer. Dark green, red, and orange circles highlight the growing cells, nongrowing/nonlysed cells, and dead cells/debris, respectively. (**b**) After a 3-h ampicillin treatment, the cells were collected, washed with PBS, and transferred to 25 mL of fresh LB media without IPTG and cultured in a shaker. Persister resuscitation was monitored at designated time points with the flow cytometer. Resuscitating cells were highlighted with green circles on flow cytometry diagrams. A representative biological replicate of flow cytometry diagrams is displayed here. All four biological replicates provideda similar result. *ONC* overnight preculture, *Exp. phase* exponential phase, *AMP* ampicillin

3.2 Setting Up the Flow Cytometry Parameters

1. Before turning on the flow cytometer, make sure the system has enough sheath, cleaning, and rinsing fluids in its containers (*see* **Note 10**).
2. Make sure the waste container is empty (*see* **Note 11**).
3. Add 50 μL of Quality Control (QC) particles to 1 mL of 1× PBS in a 5-mL round-bottom tube for a QC test (*see* **Note 12**). This leads to ~5×10^5 particles/mL.
4. Place the tube on the sample holder of the instrument and start the test. When the instrument passes the QC test, proceed with analyzing control samples (**steps 5–8**) (*see* **Note 12**).
5. Select FSC, SSC, and fluorescence parameters to be measured (*see* **Note 13**), the maximum sample volume to be analyzed, the total number of events to be collected, the sample flow rate, the core diameter, and the threshold values for FSC and SSC (*see* **Note 14**).

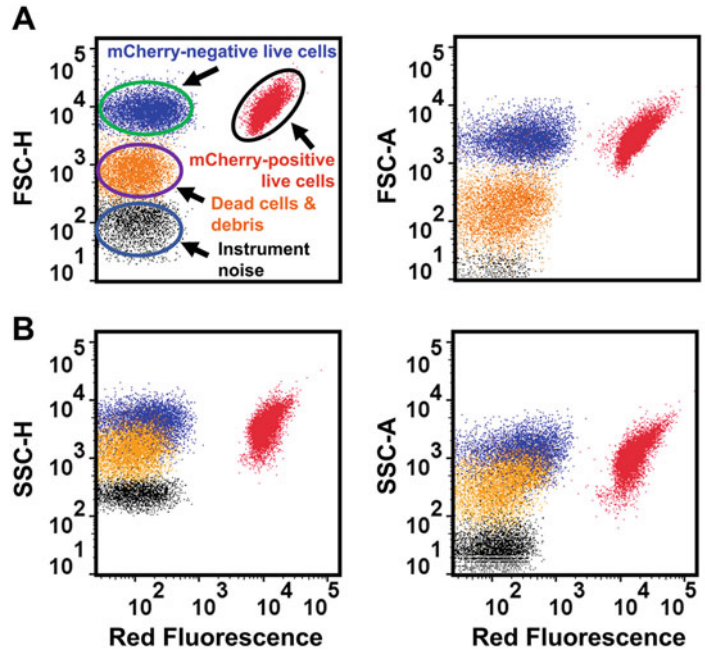

Fig. 2 Gating various subpopulations on flow cytometry diagrams. mCherry-positive and mCherry-negative live cells were diluted in PBS and analyzed with a flow cytometer to obtain their FSC, SSC and fluorescence signals. Flow data for dead cells/debris was obtained from ampicillin-treated cultures. To determine the instrument noise, 1 mL sterile PBS was also analyzed with the flow cytometer. (**a**) FSC-H or FSC-A signals of analyzed subpopulations were plotted against their red fluorescence signals. (**b**) SSC-H or SSC-A signals of analyzed subpopulations were plotted against their red fluorescence signals

6. Add 1 mL of PBS to a 5-mL tube and analyze it with the flow cytometer to determine the background noise on the flow diagram (*see* **Note 15**).
7. Dilute 10 μL of a wild-type overnight preculture (without fluorescent protein) in 1 mL of PBS in a 5-mL tube and analyze it with the flow cytometer to determine FSC, SSC and fluorescence signals of wild-type cells (Fig. 2).
8. Add 10 μL of an overnight preculture of mCherry-positive cells to 1 mL of PBS in a 5-mL tube and analyze it with the flow cytometer to determine FSC, SSC and fluorescence signals of mCherry-positive cells (Fig. 2).
9. After running the control samples described in **steps 6–8**, open density plots on the sample acquisition software.
10. Use parabolic or square shapes to gate various subpopulations (e.g., noise, mCherry-positive cells, mCherry-negative cells) on flow cytometry diagrams based on the FSC, SSC and fluorescence signals of the control samples (Fig. 2). We note that

our flow cytometer can count the number of events and measure the sample volume analyzed; this provides the fraction of a phenotypic subpopulation in a cell culture (*see* **Note 16**).

3.3 Beta-Lactam Mediated Cell Lysis

1. Dilute mCherry-positive cells from the overnight preculture 100-fold in a 250-mL Erlenmeyer flask containing 25 mL of LB medium with inducer (1 mM IPTG), and culture the cells in a shaker at 250 rpm. Prepare three identical cultures; the first one should be used to monitor beta-lactam mediated cell lysis (Subheading 3.3); the second one should be used for clonogenic survival assays (*see* Subheading 3.4) and the final one should be used to monitor persister resuscitation (*see* Subheading 3.5).
2. At desired time points, transfer 300 μL of culture from the first flask to a well of a flat-bottom 96-well plate.
3. Measure the culture absorbance (OD_{600}) with a plate reader. For background absorbance, use 300 μL of LB medium (blank). Subtract the blank absorbance from the sample absorbance to calculate the actual OD_{600}.
4. Once the culture reaches the desired growth phase (e.g., OD_{600} ~ 0.25), add a beta-lactam antibiotic (e.g., ampicillin) in all three cultures at a concentration greater than the Minimum Inhibitory Concentration (MIC) (we used 100× MIC) [26]. The MIC of ampicillin is ~6μg/mL for the *E. coli* strain we used [22].
5. At desired time points (e.g., 0, 10, 20, 30, 60, 120, 180 min), transfer 1 mL of culture from the first flask to a microcentrifuge tube.
6. Centrifuge the tube for 3 min at 17,000 × *g*.
7. Remove 950 μL of supernatant by pipetting.
8. Add 950 μL of PBS and resuspend the cell pellet. The washing procedure described in **steps 6–8** reduces the cell debris in the sample.
9. Dilute 200 μL of cell suspension from **step 8** in 800 μL of PBS in a 5-mL tube to obtain a desired cell density for flow cytometry analysis. Mix the cells thoroughly by vortexing the tube.
10. Place the tube on the sample holder of the flow cytometer and analyze it.
11. Measure SSC, FSC and red fluorescence signals as well as the number of nonlysed cells (Fig. 1a) (*see* **Note 17**).

3.4 Clonogenic Survival Assay

This assay is performed during ampicillin treatment to quantify persister cells that can form colonies on standard LB agar medium.

1. At desired time points during ampicillin treatment (e.g., 0, 1, 2, 3 h), transfer 1 mL of cell culture from the second flask (*see* **step 1** in Subheading 3.3) to a microcentrifuge tube.

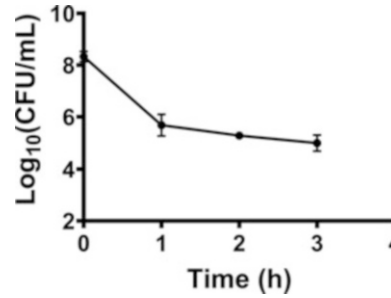

Fig. 3 Persister quantification with clonogenic survival assays. During ampicillin treatment (600μg/mL), cells were collected at designated time points, washed with PBS, and plated on agar plates to enumerate CFUs (4 biological replicates). Each data point in the figure represents mean ± standard deviation

2. Centrifuge the tube for 3 min at 17,000 × *g*.
3. Discard 950 μL of supernatant by pipetting.
4. Resuspend the pelleted cells by adding 950 μL of PBS.
5. Repeat **steps 2–4** three times in total to reduce the antibiotic concentration under the MIC.
6. After the final centrifugation, remove 900 μL of supernatant and resuspend the cell pellet in the remaining 100 μL of supernatant.
7. Mix 10 μL of cell suspension from **step 6** with 90 μL of PBS in a round-bottom 96-well plate and perform tenfold serial dilutions in PBS.
8. Plate 10 μL serially diluted cell suspensions on a solid medium (LB agar). Also, plate the remaining 90 μL undiluted cell suspension to increase the limit of detection.
9. Keep the plates inside an incubator for 16 h at 37 °C. We note that we do not observe new colonies when plates are incubated more than 16 h [22].
10. Count the colonies formed on the plates and generate kill curves by plotting the number of surviving cells as a function of time (Fig. 3).

3.5 Monitoring Persister Resuscitation

1. When the third culture is treated with ampicillin sufficiently long to kill all antibiotic-sensitive cells, transfer the entire culture to a 50-mL conical centrifuge tube. Of note, our data indicate that a 3-h ampicillin treatment is sufficient to obtain a biphasic kill curve under the conditions studied here (Fig. 3).
2. Centrifuge the tube for 15 min at 4816 × *g* at room temperature.
3. Remove the supernatant using a 25-mL serological pipette. Leave ~1 mL of supernatant in the tube.

4. Resuspend the cell pellet in the remaining 1 mL supernatant using a micropipette.
5. Transfer the cell suspension to a microcentrifuge tube.
6. Centrifuge the tube for 3 min at 17,000 × *g*.
7. Discard the supernatant.
8. Add 1 mL of PBS and resuspend the cells.
9. Repeat **steps 6–8** three times.
10. After the final centrifugation, resuspend the cells in 1 mL of fresh LB medium.
11. Transfer the 1 mL of cell suspension to a 250-mL flask containing 24 mL of LB medium (without inducer) and grow the cells in a shaker at 250 rpm.
12. At desired time points (e.g., every 30 min), take 200 μL of cells from the flask and mix with 800 μL of PBS in a 5 mL tube to obtain a desired cell density for the flow cytometry analysis.
13. Analyze the sample with the flow cytometer to measure FSC, SSC and red fluorescence signals of the nonlysed cells.
14. Gate the resuscitated cells. The fluorescent protein levels of resuscitating cells should decrease due to cell division (Fig. 1b) [36]. Also, their FSC signals should increase due to elongation characteristics of growing cells (Fig. 1b) [36].
15. Record the mean fluorescence of resuscitating cells to calculate their doubling time (*see* **Note 18**).
16. Record the number of resuscitating cells and the sample volume analyzed by the flow cytometer to calculate the resuscitating cell fractions in the culture (*see* **Note 19**).

4 Notes

1. In our experiments, we used an *E. coli* (K-12) MG1655 strain that expresses the mCherry protein in the presence of IPTG (1 mM) [21, 38, 39]. The mCherry expression system is chromosomally integrated and tightly regulated by a synthetic *T5* promoter and a $LacI^q$ repressor [21, 38, 39]. The experiments described in this chapter can also be performed using a plasmid expression system [36]. If you are planning to use a different organism, make sure that the microbial species is sensitive to the antibiotic being used.
2. Before autoclaving, mix the media very well. Select a liquid cycle for the autoclave and make sure the temperature is at least 121 °C. Prepare LB medium from its individual components, as batch to batch variation may be observed in premixed media [41].

3. If PBS is not available, use 0.85% NaCl solution for flow cytometry analysis [44]. For our experiments, we purchased filter-sterilized (0.2μm filter) 10× PBS solution. Alternatively, you can prepare the buffer solution using the method described in [45].
4. Always prepare antibiotic and IPTG solutions in aliquots (~50 μL/microcentrifuge tube) before storing at −20 °C as thawing and freezing cycles might degrade the chemicals [46, 47].
5. We used 25 mL of LB broth medium in a 250-mL flask (one-tenth of the total volume). This provides enough aeration and shaking space during culturing the cells in the flask.
6. We used pipette tips with four different volumes (2, 10, 300, and 1000 μL). Pipette tips should be autoclaved before use.
7. We used a Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific, Waltham, MA) to measure the OD_{600} in a 96-well plate format. A different instrument can be used to monitor cell growth.
8. We used a NovoCyte Flow Cytometer (NovoCyte 3000RYB, ACEA Biosciences Inc., San Diego, CA, USA).
9. NovoCyte QC particles consist of five types of polystyrene microspheres with a uniform size (average diameter of 3μm). These particles are internally stained with fluorescent dyes. Blank microspheres (without florescent dyes) are used as controls.
10. Sterile 1× PBS solution can be used as the sheath fluid but it might result in corrosion in the pump of the flow cytometer. Therefore, consult the flow cytometry specialist or vendor for appropriate sheath fluid for your instrument.
11. We add 200–300 mL concentrated bleach (100%) in the waste container for a presumptive 2 L of liquid waste.
12. A QC test is performed to assess the performance of the instrument. It is recommended to perform this test on a daily basis to evaluate the reliability of the instrument. Prepare the QC particle solution as described in the manual. Make sure to run the QC test immediately after the particle solution is prepared. One might wish to delay the QC test even though the solution is ready. In that case, make sure to store the prepared QC particles solution at 4 °C. Do not store the solution more than 4 h. The QC test run will be finished once the cytometer collects a minimum number of particles (~10,000). The report provides an overall result based on the coefficient of variance (CV) and linearity values of FSC, SSC and fluorescence parameters. If the overall result is "Pass,"

proceed with the experiment. If the outcome is "Fail," contact a flow cytometry specialist.

13. When cells pass through the laser beam, the flow cytometer detects forward (FSC) and side (SSC) scattered lights. FSC lights are proportional to the size of the cell [48, 49]. SSC lights are proportional to the shape and internal composition of the cell [48, 49]. Generally, cytoplasmic content, surface irregularities, and membrane roughness affect the SSC signal [48]. The scattered lights are converted to voltage pulses by a detector. A computer then transforms these voltage pulses to different plots (e.g., histogram, dot plot, density plot). Our instrument measures two parameters for both FSC and SSC, that is, height (H) and area (A). H represents the maximal intensity of a voltage pulse measured by the detector [48]. On the other hand, A indicates the integral of a voltage pulse over time [48]. Although we used FSC-H and SSC-H in our flow cytometry analysis, the trends from FSC-H and SSC-H data were found to be similar to those of FSC-A and SSC-A (Fig. 2).
14. Stop conditions should be specified to end the sample acquisition. We recommend to analyze at least 50,000 events or 50 μL of sample. Once either of these conditions is met, the instrument will stop analyzing the sample. A wide range of sample flow rates can be used for the flow cytometry analysis. We suggest to keep the flow rate as low as possible to achieve high resolution in data. During the analysis, cells pass through a core channel which is surrounded by sheath fluid. The sheath and core fluids flow in a coaxial manner but do not mix due to the differential pressure between these two fluids. We recommend to use a core diameter that is larger than the cell size. The threshold value indicates the lowest value of the data that will be processed and recorded for the data analysis. Appropriate threshold values will increase the efficiency of the instrument by minimizing background noise and excluding debris from the analysis [49, 50].
15. We used a logarithmic scale for the parameters to avoid background noise of the instrument while gating the analyzed cells. Since no cells are present in PBS solution, only noise will be recorded on the flow diagram. These noises can arise from the instrument voltage settings or from small particles in the medium.
16. Since our flow cytometry instrument provides both the number of cells and the sample volume being analyzed, we can readily calculate the fraction of specific cell phenotypes (e.g., VBNC cells, resuscitating cells) in cell cultures. However, if the flow cytometer does not have this option, one can use

fluorescently labeled counting beads to determine the sample volume analyzed [50]. Add an appropriate number of beads in the cell suspension and count at least 1000 beads while performing the flow cytometry analysis. As the bead concentration is known, the sample volume used by the flow cytometer can be readily determined.

17. During beta-lactam treatment, nonlysed cells can easily be identified on the flow diagram. Fluorescence signals of nonlysed cells remain constant; however antibiotic sensitive cells lose their membrane integrity and fluorescent proteins. With live–dead staining, fluorescence-activated cell sorting (FACS), clonogenic survival assays and metabolic measurements, we have previously verified that nonlysed cell subpopulations are enriched with persister and VBNC cells [38, 39].
18. We calculate the doubling time of growing cells using the exponential decay equation, $R = R_o 2^{-(t-t_o)/t_d}$, where R_o is the initial mean fluorescence level of the resuscitating cells at time t_o, R is the mean fluorescence level at time t, and t_d is the doubling time [36].
19. A traditional doubling time equation can be used to calculate the number of cells resuscitated, $N = N_o 2^{(t-t_o)/t_d}$, where N_o is the initial number of resuscitated cells at t_o, N is the number of resuscitated cells at time t, and t_d is the doubling time [36].

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References

1. Hobby GL, Meyer K, Chaffee E (1942) Observations on the mechanism of action of penicillin. Exp Biol Med 50:281–285
2. Bigger JW (1944) Treatment of staphylococcal infections with penicillin by intermittent sterilisation. Lancet 244:497–500
3. Van den Bergh B, Fauvart M, Michiels J (2017) Formation, physiology, ecology, evolution and clinical importance of bacterial persisters. FEMS Microbiol Rev 41:219–251
4. Balaban NQ, Merrin J, Chait R et al (2004) Bacterial persistence as a phenotypic switch. Science 305:1622–1625
5. Lewis K (2010) Persister cells. Annu Rev Microbiol 64:357–372
6. Harms A, Maisonneuve E, Gerdes K (2016) Mechanisms of bacterial persistence during stress and antibiotic exposure. Science 354: aaf4268
7. Völzing KG, Brynildsen MP (2015) Stationary-phase persisters to ofloxacin sustain DNA damage and require repair systems only during recovery. mBio 6:e00731-15
8. Dörr T, Lewis K, Vulić M (2009) SOS response induces persistence to fluoroquinolones in *Escherichia coli*. PLoS Genet 5:e1000760

9. Hansen S, Lewis K, Vulić M (2008) Role of global regulators and nucleotide metabolism in antibiotic tolerance in *Escherichia coli*. Antimicrob Agents Chemother 52:2718–2726
10. Nguyen D, Joshi-Datar A, Lepine F et al (2011) Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. Science 334:982–986
11. Amato SM, Orman MA, Brynildsen MP (2013) Metabolic control of persister formation in *Escherichia coli*. Mol Cell 50:475–487
12. Moyed HS, Bertrand KP (1983) *hipA*, a newly recognized gene of *Escherichia coli* K-12 that affects frequency of persistence after inhibition of murein synthesis. J Bacteriol 155:768–775
13. Rotem E, Loinger A, Ronin I et al (2010) Regulation of phenotypic variability by a threshold-based mechanism underlies bacterial persistence. Proc Natl Acad Sci U S A 107:12541–12546
14. Schumacher MA, Piro KM, Xu W et al (2009) Molecular mechanisms of HipA-mediated multidrug tolerance and its neutralization by HipB. Science 323:396–401
15. Korch SB, Henderson TA, Hill TM (2003) Characterization of the *hipA7* allele of *Escherichia coli* and evidence that high persistence is governed by (p)ppGpp synthesis. Mol Microbiol 50:1199–1213
16. Cirillo SLG, Subbian S, Chen B et al (2009) Protection of *Mycobacterium tuberculosis* from reactive oxygen species conferred by the *mel2* locus impacts persistence and dissemination. Infect Immun 77:2557–2567
17. Grant SS, Kaufmann BB, Chand NS et al (2012) Eradication of bacterial persisters with antibiotic-generated hydroxyl radicals. Proc Natl Acad Sci U S A 109:12147–12152
18. Wu Y, Vulić M, Keren I et al (2012) Role of oxidative stress in persister tolerance. Antimicrob Agents Chemother 56:4922–4926
19. Dörr T, Vulić M, Lewis K (2010) Ciprofloxacin causes persister formation by inducing the TisB toxin in *Escherichia coli*. PLoS Biol 8:e1000317
20. Fung DKC, Chan EWC, Chin ML et al (2010) Delineation of a bacterial starvation stress response network which can mediate antibiotic tolerance development. Antimicrob Agents Chemother 54:1082–1093
21. Orman MA, Brynildsen MP (2015) Inhibition of stationary phase respiration impairs persister formation in *E. coli*. Nat Commun 6:1–13
22. Mohiuddin SG, Hoang T, Saba A et al (2020) Identifying metabolic inhibitors to reduce bacterial persistence. Front Microbiol 11:472
23. Orman MA, Brynildsen MP (2016) Persister formation in *Escherichia coli* can be inhibited by treatment with nitric oxide. Free Radic Biol Med 93:145–154
24. Shan Y, Gandt AB, Rowe SE et al (2017) ATP-dependent persister formation in *Escherichia coli*. mBio 8:e02267-16
25. Karki P, Mohiuddin SG, Kavousi P et al (2020) Investigating the effects of osmolytes and environmental pH on bacterial persisters. Antimicrob Agents Chemother 64:e02393–e02319
26. Keren I, Kaldalu N, Spoering A et al (2004) Persister cells and tolerance to antimicrobials. FEMS Microbiol Lett 230:13–18
27. Roostalu J, Jõers A, Luidalepp H et al (2008) Cell division in *Escherichia coli* cultures monitored at single cell resolution. BMC Microbiol 8:68
28. Barrett TC, Mok WWK, Murawski AM et al (2019) Enhanced antibiotic resistance development from fluoroquinolone persisters after a single exposure to antibiotic. Nat Commun 10:1–11
29. Yamasaki R, Song S, Benedik MJ et al (2020) Persister cells resuscitate using membrane sensors that activate chemotaxis, lower cAMP levels, and revive ribosomes. iScience 23:100792
30. Kim J, Yamasaki R, Song S et al (2018) Single cell observations show persister cells wake based on ribosome content. Environ Microbiol 20:2085–2098
31. Wilmaerts D, Dewachter L, De Loose PJ et al (2019) HokB monomerization and membrane repolarization control persister awakening. Mol Cell 75:1031–1042.e4
32. Svenningsen MS, Veress A, Harms A et al (2019) Birth and resuscitation of (p)ppGpp induced antibiotic tolerant persister cells. Sci Rep 9:1–13
33. Windels EM, Ben MZ, Zahir T et al (2019) Enrichment of persisters enabled by a β-lactam-induced filamentation method reveals their stochastic single-cell awakening. Commun Biol 2:1–7
34. Goormaghtigh F, Van Melderen L (2019) Single-cell imaging and characterization of *Escherichia coli* persister cells to ofloxacin in exponential cultures. Sci Adv 5:eaav9462
35. Jõers A, Kaldalu N, Tenson T (2010) The frequency of persisters in *Escherichia coli* reflects the kinetics of awakening from dormancy. J Bacteriol 192:3379–3384
36. Mohiuddin SG, Kavousi P, Orman MA (2020) Flow-cytometry analysis reveals persister resuscitation characteristics. BMC Microbiol 20:202
37. Ayrapetyan M, Williams T, Oliver JD (2018) Relationship between the viable but

nonculturable state and antibiotic persister cells. J Bacteriol 200:e00249–e00218
38. Orman MA, Brynildsen MP (2013) Establishment of a method to rapidly assay bacterial persister metabolism. Antimicrob Agents Chemother 57:4398–4409
39. Orman MA, Brynildsen MP (2013) Dormancy is not necessary or sufficient for bacterial persistence. Antimicrob Agents Chemother 57:3230–3239
40. Keren I, Shah D, Spoering A et al (2004) Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. J Bacteriol 186:8172–8180
41. Nikaido H (2009) Small things considered: the limitations of LB medium. https://schaechterasmblogorg/schaechter/2009/11/the-limitations-of-lb-mediumhtmL. Accessed 15 Sept 2020
42. Veal DA, Deere D, Ferrari B et al (2000) Fluorescence staining and flow cytometry for monitoring microbial cells. J Immunol Methods 243:191–210
43. Suller MTE, Lloyd D (1999) Fluorescence monitoring of antibiotic-induced bacterial damage using flow cytometry. Cytometry 35:235–241
44. Khalili B, Weihe C, Kimball S et al (2019) Optimization of a method to quantify soil bacterial abundance by flow cytometry. mSpere 4: e00435-19
45. (2001) Common media, buffers, and stock solutions. Curr Protoc Immunol 34: A.2A.1–A.2A.8
46. Fernández A, Ruiz J, Caminal G et al (2010) Development and validation of a liquid chromatography-mass spectrometry assay for the quantitation of IPTG in *E. coli* fed-batch cultures. Anal Chem 82:5728–5734
47. Andrews JM (2001) Determination of minimum inhibitory concentrations. J Antimicrob Chemother 48:5–16
48. Tzur A, Moore JK, Jorgensen P et al (2011) Optimizing optical flow cytometry for cell volume-based sorting and analysis. PLoS One 6:16053
49. Cossarizza A, Chang HD, Radbruch A et al (2019) Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). Eur J Immunol 49:1457–1973
50. Strauss K, Hannet I, Engels S et al (1996) Performance evaluation of the FACSCount system: a dedicated system for clinical cellular analysis. Commun Clin Cytom 26:52–59

Chapter 15

Stimulating Aminoglycoside Uptake to Kill *Staphylococcus aureus* Persisters

Ashelyn E. Sidders, Lauren C. Radlinski, Sarah E. Rowe, and Brian P. Conlon

Abstract

Aminoglycosides are bactericidal drugs which require a proton motive force (PMF) for uptake into the bacterial cell. Low energy cells, such as persisters, maintain a PMF below the threshold for drug uptake and are tolerant to aminoglycosides. In this chapter, we discuss mechanisms to target the bacterial membrane and stimulate aminoglycoside uptake to kill *Staphylococcus aureus* persisters.

Key words *Staphylococcus aureus*, Aminoglycosides, Adjuvants, Synergy, Persisters

1 Introduction

Antibiotic treatment of *S. aureus* fails in approximately one in five patients, leading to an estimated 20,000 deaths annually in the USA alone [1]. Conventional views surmise that antibiotic resistance is the primary cause of treatment failure. However, a proportion of clinical isolates that fail to be cleared by antibiotics often exhibit full sensitivity to the administered drug when tested in vitro using a minimum inhibitory concentration (MIC) assay, and several studies have shown MIC testing is a poor predictor of treatment outcome [2–4]. These observations indicate that treatment failure is a complex, multifaceted issue that cannot be fully attributed to resistance. As such, there is a growing appreciation for the role of antibiotic tolerant persister cells in treatment outcome [5–7].

Antibiotic tolerance can be defined as the ability to survive lethal concentrations of bactericidal antibiotics without an increased MIC; persister cells are defined as a subpopulation of tolerant cells within an otherwise susceptible bacterial population [8]. The persister fraction survives high concentrations of bactericidal antibiotics that target active cellular processes by entering a

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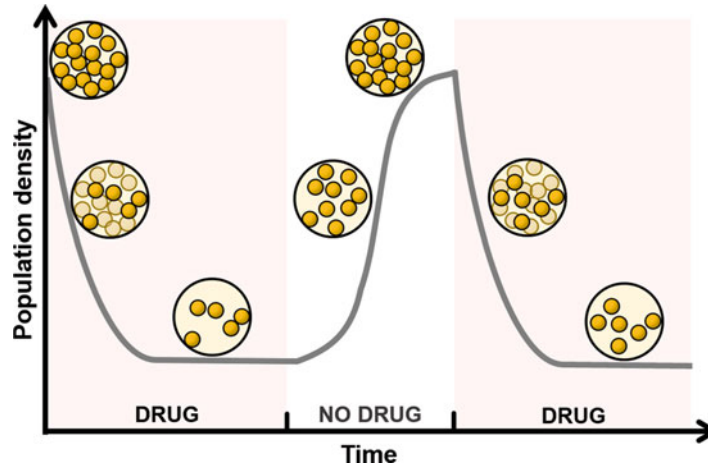

Fig. 1 Graphical representation of *S. aureus* persisters. When a *S. aureus* population is exposed to high concentrations of a bactericidal antibiotic, the susceptible population is rapidly killed by the drug. However, within this population of *S. aureus* there is a fraction of cells known as persisters that neither grow nor die despite the presence of lethal drug concentrations. The persister fraction is visualized as the plateau of a biphasic kill curve. Once the drug is removed from the environment, the persister cells resuscitate from a low energy state and regrow. Because the bacterial population is not resistant to the antibiotic, a second round of antibiotic challenge will kill susceptible cells while leaving behind the persister fraction

low energy or metabolically quiescent state [9, 10]. The fraction of *S. aureus* persisters can be quantified by challenging an exponentially growing culture with lethal concentrations of a bactericidal antibiotic and enumerating survivors at various time points. The resulting data generates a distinct biphasic kill curve where susceptible cells die rapidly after antibiotic challenge and the persister fraction neither grows nor dies over time (Fig. 1). In the context of patient health, this surviving fraction of persisters can resume growth upon cessation of treatment, leading to chronic and relapsing *S. aureus* infections [6]. Additionally, recent work has suggested that antibiotic tolerance and persistence precedes and facilitates the evolution of antibiotic resistant strains [11, 12]. As such, the concerning rise in multidrug-resistant bacterial infections coupled with an evaporating pipeline of new antibiotics reaching the market, emphasizes the importance of developing strategies to enhance the efficacy of our current arsenal of antibiotics against tolerant and resistant bacterial populations.

Among the antibiotics that exemplify the disconnection between MIC and treatment outcome are broad-spectrum aminoglycosides. This class of antimicrobials have a mechanism of action that occurs in two phases for bactericidal activity. The initial phase is aminoglycoside diffusion into the cell by electrostatic interactions

with the cell surface, followed by a proton motive force (PMF) dependent uptake of aminoglycosides to reach the cytoplasmic target [13, 14]. Aminoglycosides bind to the 30S ribosomal subunit promoting tRNA mismatching, which leads to protein misfolding and the production of toxic peptides. Toxic peptides are thought to insert into the cell membrane, thereby enhancing permeability which permits an exponential increase of drug influx (second phase of drug uptake) resulting in cell death [15, 16]. This class of antibiotics is very effective against respiring cells which maintain a PMF at sufficient levels to facilitate drug uptake. However, aminoglycosides have limited utility against tolerant populations such as persister cells, biofilm cells, small colony variants or cells undergoing fermentation which maintain a PMF below the threshold required for drug uptake (Fig. 2) [17, 18].

In an effort to revitalize this class of antibiotics against difficult-to-treat *S. aureus* infections, several membrane-acting compounds have been identified that synergize with aminoglycosides including the retinoid adarotene and the monoglyceride glycerol monolaurate (GML) [19–21]. Our group has recently identified rhamnolipids (RLs), biosurfactants produced by *Pseudomonas aeruginosa*, as potent aminoglycoside adjuvants [22, 23]. To unravel the mechanism of aminoglycoside synergy, we have identified a concentration of each putative adjuvant (adarotene, GML, and RLs) that potentiates aminoglycoside killing without exerting bactericidal activity alone and evaluated their impact on *S. aureus*. Although we found that all three adjuvants potentiate aminoglycoside killing of

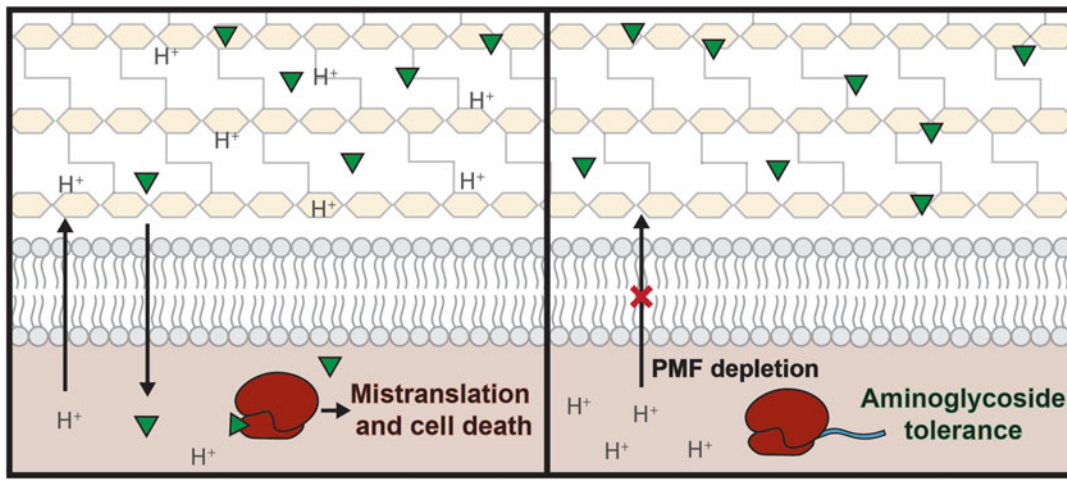

Fig. 2 Aminoglycoside efficacy under various conditions. Aminoglycosides such as tobramycin are potent antibiotics against respiring or metabolically active bacteria that maintain an active proton motive force (PMF) that allows the drug to readily diffuse into the cell. In contrast, aminoglycosides have negligible efficacy against nonrespiring or dormant bacterial populations that maintain a PMF below the threshold required for drug uptake

S. aureus persister cells, only RLs could restore antibiotic efficacy when we induced antibiotic tolerance of the population by pretreating cells with arsenate to diminish intracellular ATP levels or by collapsing PMF using a proton ionophore. Furthermore, only RLs could enhance the initial phase of aminoglycoside influx into the cell [23]. These data indicate that adarotene and GML synergy likely enhances the final phase of aminoglycoside activity by increasing membrane destabilization downstream of PMF-dependent drug penetration, limiting their utility against important infection niches and suggesting the primary barrier to aminoglycoside efficacy lies in drug uptake. In summary, adjuvants that stimulate the initial phase of aminoglycoside uptake represent an ideal strategy to revitalize this class of antibiotics as we have shown that RLs (1) repress the rise of resistance, (2) restore susceptibility of highly aminoglycoside resistant strains, and (3) rapidly eradicate recalcitrant bacterial populations including persisters [23].

We speculate that this mechanism of potentiation is not unique to RLs and other compounds may act similarly. Here we outline methods to delineate whether a compound synergizes with aminoglycosides by promoting initial uptake to kill persisters or acts downstream of drug influx.

2 Materials

Unless otherwise stated, prepare all reagents and media using deionized water. Store liquid media and agar plates at room temperature. If agar plates need to be stored for longer than a few days, store at 4 °C, agar side up, in an airtight bag.

1. Mueller-Hinton Broth (MHB) medium: Dissolve powder in water according to the manufacturer's directions. Sterilize by autoclaving and allow the media to cool before using (*see* **Note 1**).
2. Tryptic soy broth (TSB) agar plates: Dissolve TSB powder in water according to the manufacturer's directions. Dissolve agar (1% w/v) and sterilize by autoclaving. Allow media to cool to ~50 °C prior to pouring into petri dishes. Allow agar to cool until solidified (*see* **Note 2**).
3. 1% NaCl: Dissolve NaCl into water and sterilize by autoclaving.
4. Tobramycin: Make a 50 mg/mL stock solution by dissolving tobramycin powder into water. Store aliquots at −20 °C. Freeze and thaw aliquots once, then discard.
5. Texas Red-Succinimidyl ester (Texas Red): Make a 20 mg/mL stock solution by dissolving Texas Red powder into high-quality anhydrous *N,N*-dimethylformamide. Store at −20 °C and minimize exposure to light.

6. Potassium carbonate buffer: Dissolve 1.38 g of K_2CO_3 powder into 100 mL of water for a 100 mM final concentration and pH solution to pH 8.5. Filter sterilize and store at room temperature.
7. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP): Make a 1 mM stock of CCCP by dissolving powder into DMSO. Aliquot and store at −20 °C. Shield from light. Allow aliquot to come to room temperature before use.
8. Potassium arsenate monobasic (arsenate): Make a 5 M stock of arsenate in water. Store at −20 °C.

3 Methods

3.1 Quantifying Persister Cells

1. Under sterile conditions, streak out the *S. aureus* strain of interest from a frozen glycerol stock on to an agar plate. Incubate the plate overnight at 37 °C. Store at 4 °C the following day for further use (make a freshly streaked plate weekly).
2. Inoculate a sterile culture tube containing 3 mL of MHB medium with cells from a single colony. Repeat twice to generate a total of three independent cultures.
3. Incubate cultures overnight (~18 h) on a shaker at 225 rpm and 37 °C.
4. For each condition, prepare three 14 mL capped culture tubes (one for each biologically independent overnight culture) with 3 mL of MHB medium. Dilute the overnight cultures 1:100 into the respective culture tubes.
5. Incubate diluted cultures on a shaker at 225 rpm and 37 °C until they reach ~3×10^8 CFU/mL (*see* **Note 3**). After the time required to reach this density, vortex each culture briefly and take 100 μL of each to determine the number of culturable bacteria prior to aminoglycoside challenge. Set aside until **step 7**.
6. Thaw a tobramycin stock solution aliquot and dilute in water to create a working solution if necessary. Add tobramycin to the culture to achieve a final concentration of 20× MIC of tobramycin (*see* **Note 4**). Continue incubation of cultures on a shaker at 225 rpm and 37 °C.
7. Make serial dilutions of the aliquots taken in **step 5** prior to treatment by using 1% NaCl for dilutions (*see* **Note 5**).
8. Spot plate 10 μL of serial dilutions starting from the lowest concentration onto an agar plate (*see* **Note 6**). Keep plates upright until the spots on the agar surface are completely dry to avoid merging of spots. Subsequently, place inverted plates at 37 °C and incubate overnight (*see* **Note 7**).

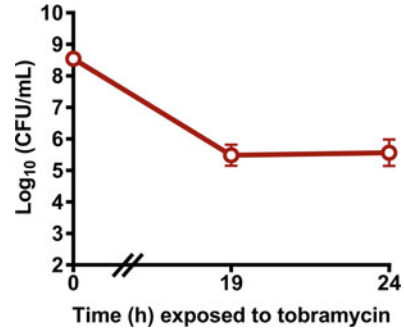

Fig. 3 Representative tobramycin biphasic kill curve of *S. aureus*. *S. aureus* HG003 cultures were grown to ~3 × 10^8 CFU/mL prior to treatment with tobramycin at 20× MIC (15.6 μg/mL). For each time point an aliquot of cells was removed, washed, and plated to visualize the persister plateau. Data represents the average of three biologically independent replicates

9. When using 20× MIC of tobramycin, take 100 μL aliquot of each culture (**step 6**) at 19 and 24 h after addition of the drug and dispense into labeled, sterile 1.5 mL microcentrifuge tubes (*see* **Note 8**).
10. Spin down the cells for 2 min in a tabletop microcentrifuge at 9600 × *g* at room temperature. Remove the supernatant and resuspend cells in 100 μL of 1% NaCl. Repeat the washing step once more. Perform serial dilution steps as stated in **steps 7** and **8** for each time point.
11. Incubate plates overnight (*see* **Note 7**) and count the dilution for each culture that has between 10–100 colonies. An example of the resulting persister fraction is shown in Fig. 3.

3.2 Determining Adjuvant Concentration

1. Follow **steps 1–3** outlined in Subheading 3.1.
2. For each full 96-well plate that will be utilized in the screen, dilute the overnight culture 1:100 into a sterile 100 mL flask containing 10 mL of fresh MHB.
3. Allow the culture to grow to ~3 × 10^8 CFU/mL.
4. Meanwhile, prepare a 96-well plate with a range of concentrations by performing two-fold serial dilutions of the putative aminoglycoside adjuvant ± tobramycin at 10× above the MIC (*see* **Note 9**).
5. Once the culture reaches the desired starting CFU/mL, add 100 μL of *S. aureus* culture to columns 1–11 of rows A–F (*see* **Note 10**). Incubate the 96-well plate on a shaker for 24 h at 37 °C (*see* **Note 11**).
6. After 24 h, spin the plates for 10 min at 9600 × *g* at room temperature to pellet the cells, wash cell pellets 2× in 200 μL of 1% NaCl, resuspend in 200 μL of 1% NaCl, perform ten-fold

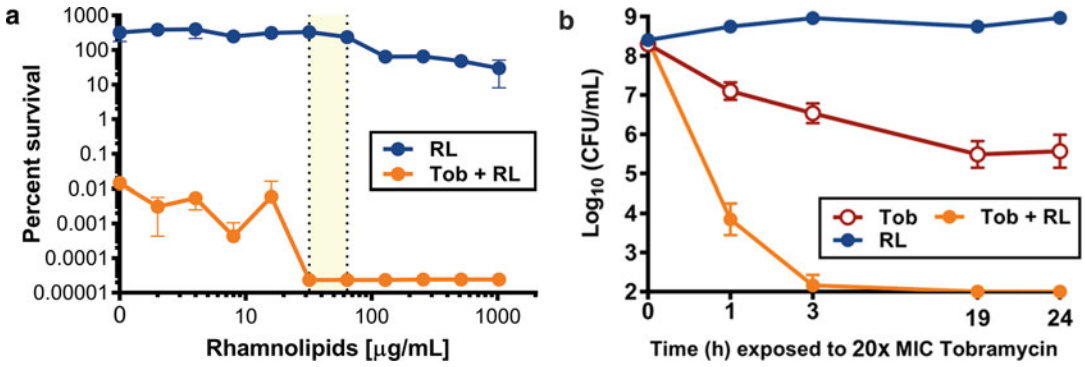

Fig. 4 Identifying a sublethal concentration of a putative adjuvant that retains the capacity to potentiate killing. *S. aureus* HG003 was grown to ~3 × 10^8 CFU/mL in MHB. (**a**) HG003 was then added to a 96-well plate preloaded with two-fold dilutions of RL ± 10× MIC tobramycin (7.8 μg/mL). After 24 h of treatment, the 96-well plate was pelleted, washed, and plated to enumerate survivors. For all data points, percent survival was calculated relative to starting CFU prior to treatment. The blue circles represent percent survival of each two-fold dilution of RL alone, while the orange circles represent combination treatment of tobramycin with each dilution of RL tested. The yellow shaded area between two dashed lines is the concentration range (30–60 μg/mL) of RL that showed no decrease in cell viability compared to the untreated control (first blue data point) and potentiated tobramycin killing compared to tobramycin alone (first orange data point). (**b**) Using the protocol outlined in Subheading 3.1, the lowest RL concentration (30 μg/mL) was further confirmed to be sublethal alone while still potentiating tobramycin killing, eradicating the population down to the limit of detection

serial dilutions in 1% NaCl, spot-plate 10 μL of each dilution and incubate plates for colony counting as stated in **step 8** of Subheading 3.1. An example using RLs is shown in Fig. 4a.

7. From these plate-based assays identify a range of concentrations (~3) or a single concentration of adjuvant that potentiates tobramycin killing without bactericidal activity in the absence of tobramycin (*see* **Note 12**).
8. To confirm that the determined concentration(s) from the plate-based assay show the same characteristics in a kill curve using culture tubes, follow the protocol outlined in Subheading 3.1 and use the following conditions: (a) untreated control, (b) each concentration of adjuvant alone, (c) 20× MIC of tobramycin alone, and (d) each concentration of adjuvant in combination with tobramycin.
9. Prior to adjuvant ± antibiotic challenge and at 1, 3, 5, and 24 h after treatment, take time points as follows: wash, serially dilute, and plate to enumerate survivors as indicated in Subheading 3.1. In future experiments, use the highest concentration of adjuvant that does not significantly kill *S. aureus* on its own, while still significantly potentiating tobramycin killing as illustrated in Fig. 4b.

3.3 Evaluating Killing Against Chemically Induced Tolerant S. aureus

1. For each compound of interest you will have the following conditions: (a) the compound alone, (b) tobramycin alone, (c) CCCP alone, (d) arsenate alone, (e) compound + tobramycin, (f) tobramycin + CCCP, (g) tobramycin + arsenate, (h) compound + tobramycin + CCCP, (i) compound + tobramycin + arsenate (*see* **Note 13**).
2. Prepare three culture tubes for each condition with 3 mL of MHB (*see* **steps 1–3** in Subheading 3.1) and dilute the overnight cultures 1:100 into 3 mL of fresh medium. Incubate on a shaker at 225 rpm and 37 °C.
3. For the conditions that include CCCP or arsenate, 30 min prior to the determined incubation period necessary for a culture to reach ~3 × 10^8 CFU/mL, add 1 μM CCCP or 5 mM arsenate to the respective culture tubes.
4. After pretreatment with CCCP/arsenate, take your starting time point prior to adding adjuvant ± tobramycin, vortex each culture and take 100 μL for serial dilution and plating as described in Subheading 3.1.
5. For conditions outlined in **step 1** of Subheading 3.3, add 20× MIC of a fresh tobramycin working solution as described in Subheading 3.1 and add the sublethal concentration of adjuvant determined in Subheading 3.2.
6. As mentioned in Subheading 3.2, when challenging cultures with 20× MIC of tobramycin in combination with a potent adjuvant, we have seen rapid sterilization of the culture. As such, survivors are enumerated at early and late time points (we typically use 1, 3, 19, and 24 h) post antibiotic treatment to visualize whether the adjuvant can sensitize an otherwise tolerant population to tobramycin killing. At these time points, a 100 μL aliquot if removed, washed, serially diluted, plated, and incubated overnight. An example of PMF-independent potentiation by adding RL is presented in Fig. 5.

3.4 Texas Red-Conjugated Tobramycin Uptake

1. For each putative aminoglycoside adjuvant to be tested you will have the following conditions: (a) tobramycin alone, (b) tobramycin + CCCP, (c) putative adjuvant + tobramycin (*see* **Note 14**).
2. Prepare three culture tubes for each condition with 3 mL of MHB (**steps 1–3** in Subheading 3.1) and dilute the overnight cultures 1:100 into 3 mL of fresh medium. Incubate on a shaker at 225 rpm and 37 °C.
3. Meanwhile, dissolve 10 mg of tobramycin into 1 mL of 100 mM K_2CO_3, pH 8.5 to yield a final concentration of 10 mg/mL. Place on ice.

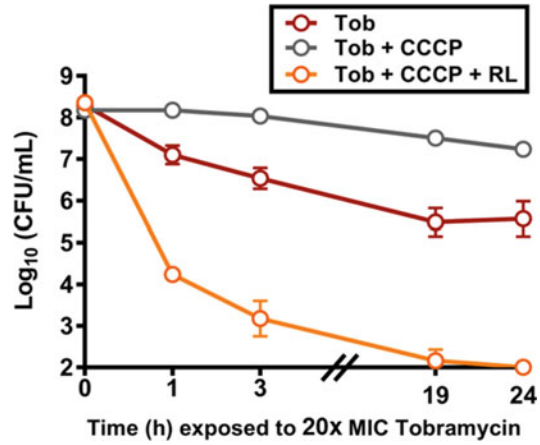

Fig. 5 Sensitizing PMF-depleted *S. aureus* to tobramycin. To collapse the PMF of *S. aureus* HG003, cultures were pretreated with 1 μM CCCP for 30 min prior to reaching ~3 × 10^8 CFU/mL. At time points indicated an aliquot of cells was removed, washed, and serial dilutions were plated to enumerate survivors. The grey line illustrates PMF-depleted *S. aureus* populations that are protected from tobramycin killing due to diminished drug uptake. However, the addition of RL (orange line) sensitizes PMF-depleted *S. aureus* to killing by tobramycin. These results are representative of an adjuvant that overcomes the requirement of PMF for drug influx, the primary barrier in aminoglycoside efficacy

4. On ice, slowly add 28.6 μL of the Texas Red stock solution to 1 mL of the 10 mg/mL tobramycin solution (*see* **Note 15**).
5. As described in **step 3** of Subheading 3.3, pretreat culture tubes that receive CCCP for 30 min prior to tobramycin treatment.
6. After 30 min pretreatment when cultures reach ~3 × 10^8 CFU/mL, vortex each culture and take 100 μL for serial dilution and plating as described in Subheading 3.1.
7. To each condition, add Texas Red–conjugated tobramycin for a final concentration of 20× MIC. Continue to incubate on a shaker at 225 rpm at 37 °C. Cover the tubes with aluminum foil to shield from light.
8. To measure initial uptake of tobramycin, remove 100 μL from the cultures at 0.5 and 1 h after Texas Red-tobramycin challenge and wash two times with 1% NaCl as described in Subheading 3.1.
9. Dispense 0.75 mL of 1% NaCl into flow cytometry compatible tubes and add 30 μL of cells from washed samples into the tubes. Use the remaining washed cells to perform serial dilutions and plating to quantify survivors.
10. Analyze Texas-red fluorescence by flow cytometry. Compare fluorescence of each drug-treated sample to a CCCP-treated

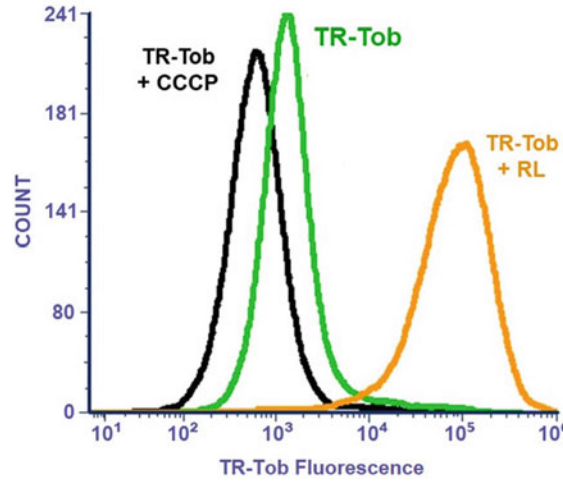

Fig. 6 Measuring initial Texas Red–conjugated tobramycin uptake using flow cytometry. The black curve corresponds to *S. aureus* cells that were pretreated with CCCP to deplete the PMF prior to treating with Texas Red-conjugated tobramycin (TR-Tob); this represents the negative control as TR-Tob is excluded entirely. In green are cells treated with TR-Tob alone, while orange represents combination therapy of RL + TR-Tob. All cells were taken after a single hour of treatment to visualize uptake. Although TR-Tob alone (green) shows a slight shift in fluorescence compared to the negative control (black), the drastic shift of antibiotic-adjuvant combination (orange) compared to monotherapy (green) illustrates a significant increase in TR-Tob uptake after a short time interval. Uptake was measured by recording 30,000 events using an Attune NxT flow cytometer. Histogram of data gathered was generated by FCS Express 6 Flow. The *X*-axis indicates Texas Red fluorescence

sample and an unstained sample (*see* **Note 16**). Record at least 30,000 events for each sample.

11. Overlay histograms to compare Texas Red fluorescence as in Fig. 6 or present data as a bar graph of mean fluorescence intensity for each biological replicate and each condition.

4 Notes

1. Persister frequency is dependent on numerous factors including growth medium [24]. We have observed variability between brands of MHB and thus we recommend utilizing a single brand that leads to reproducible results. In our lab, we utilize Oxoid MHB.
2. Persisters recover faster on rich media [24] so following antibiotic challenge and washing, serial dilutions are plated on tryptic soy broth (TSB) or brain heart infusion (BHI) agar plates. For best results, ensure plates dry at room temperature for at least 48 h prior to using to plate serial dilutions.

3. Susceptibility to aminoglycosides varies dramatically between strains. Typically, to reach ~3 × 10^8 CFU/mL with our laboratory strain HG003 [25], we allow the culture to grow for ~4 h. However, we recommend that each lab optimizes the amount of time required to reach ~3 × 10^8 CFU/mL and considers increasing or decreasing the density to achieve the desired aminoglycoside killing for the strain of interest [26].
4. At high concentrations (above 50× MIC), aminoglycosides kill over 99.99% of exponentially growing *S. aureus* cells which leaves little room for identifying adjuvants that enhance killing [17]. Therefore, 20× MIC is more suitable for identifying potential adjuvants.
5. Alternatively, PBS can be used for dilutions. In our lab we utilize a 96-well plate format for our serial dilutions and an automatic multichannel pipette (Eppendorf, Item # UX-24500-88) for 10× serial dilutions. Furthermore, our setup for serial dilutions is as follows.
   (a) Add 100 μL of culture to row A of a microtiter plate.
   (b) Add 90 μL of 1% NaCl to rows B–F.
   (c) Transfer 10 μL from row A into row B. Mix several times by gently pipetting.
   (d) Discard tips from your multichannel pipette and attach new sterile tips prior to each dilution.
   (e) Repeat **steps** (**c**) and (**d**) until you complete all serial dilutions down to row F.
6. We use a multichannel pipette to spot plate dilutions and typically plate six columns and three rows of 10 μL on a single plate. For example, six tips are attached to a multichannel and 10 μL of the row F dilution (columns 1–6) is aspirated. While dispensing the 10 μL, slowly move the multichannel forward to create a roughly inch-long line of cells onto an agar plate. Alternatively, you can dispense the 10 μL and immediately tilt the plate to allow the cells to spread. Ensure that there is sufficient space for additional dilutions (rows) of the same columns to fit on the agar plate. Starting from the most diluted row of cells makes it unnecessary to change tips between dilutions (rows).
7. In some cases, persisters exposed to membrane acting agents and/or antibiotic challenge take longer to recover. In these cases, allow persisters 24–48 h to regrow before counting.
8. Consistently, our group finds the characteristic persister plateau present between 16–24 h post aminoglycoside treatment. To visualize the plateau, two time points within this period are required. However, when measuring synergy with membrane acting agents in this assay, we frequently see killing to the limit

of detection occurs rapidly and additional early time points should be taken between 1 and 6 h [22, 23].

9. In a 96-well plate, rows A–D represent the four technical replicates measuring the bactericidal activity of the putative aminoglycoside adjuvant alone, while rows E–H represent the four technical replicates that are challenged with the same adjuvant concentration range in combination with 10× MIC of tobramycin. It is important to note that some membrane acting compounds may precipitate out of solution or form micelles if the starting concentration is too high. If these phenomena occur, reduce the starting concentration. To setup the 96-well plate, perform the following.
   (a) Create a 4× stock solution in MHB of the highest adjuvant concentration to be tested and aliquot 100 μL of stock solution into column 1 for all rows.
   (b) Add 50 μL of MHB to column 2–11 for all rows.
   (c) Perform two-fold serial dilutions by transferring 50 μL from column 1 to column 2 and mix by gently pipetting up and down. Continue two-fold serial dilutions until column 10 and discard the remaining 50 μL from column 10.
   (d) For rows A–D, add 50 μL of MHB to columns 1–11.
   (e) Make a 4× tobramycin stock solution (31.2 μg/mL) in MHB that will be diluted to 10× MIC.
   (f) For rows E–H, add 50 μL of tobramycin stock solution to columns 1–11.
   (g) Add 200 μL of MHB to column 12 to serve as a medium control.
   (h) Finally, add 100 μL of culture that was grown to ~3 × 10^8 CFU/mL to columns 1–11 for all rows. Ensure you enumerate the starting CFU/mL in order to calculate percent survival after challenge.
10. Since the starting CFU/mL is two-fold lower than what is utilized in Subheading 3.1, we found using 10× instead of 20× MIC of tobramycin yields more consistent data. Column 11 of rows A–D serves as the untreated control to determine if any concentration within the range of adjuvant tested has bactericidal activity. Column 11 of rows E–H serves as a control to measure bactericidal activity of tobramycin monotherapy.
11. Due to the small volume of liquid in each well, most plate-based assays incubated at 37 °C for long periods can result in evaporation. To prevent evaporation, we use a gas permeable seal and a digital microplate shaker in a smaller tabletop incubator with humidity control.
12. When membrane-acting agents are studied at bactericidal concentration, their mechanism may be associated with a broad

panel of effects making it challenging to determine which alterations to the cell are associated with synergy and which are simply a consequence of cell death. Therefore, it is helpful to examine potentiation at sublethal adjuvant concentrations over short treatment times to gain insight in the mechanisms that lead to synergy.

13. CCCP is a protonophore and uncoupling agent which collapses the PMF and prevents tobramycin uptake. Only compounds that overcome the requirement for PMF-dependent influx of tobramycin will restore aminoglycoside activity when CCCP is present. Arsenate reduces the intracellular ATP levels of the cell [10, 23]. Only compounds that lower the threshold energy and translation levels required for killing by aminoglycosides will be able to restore aminoglycoside activity. For a positive control for potentiation under these conditions, you can include RL90 (AGAE) at a final concentration of 30 μg/mL.
14. CCCP-treated cells will be considered the negative control as tobramycin is excluded. However, at early time points such as 0.5 or 1 h, the negative control may look similar to tobramycin alone as drug uptake at these time points is minimal even without CCCP. For a positive control you can again utilize RL90 at a final concentration of 30 μg/mL.
15. To maximize the formation of Texas Red-conjugated tobramycin, it is essential that the conjugation reaction utilizes tobramycin at least at a 30 molar excess [27]. Slowly add Texas Red by adding 2 μL of Texas Red stock solution to tobramycin, invert, add another 2 μL, then invert, and repeat until a total of 20 μL has been added. Make right before using then discard. Ensure that Texas Red-tobramycin is always kept on ice and protected from light.
16. Take note of the event/s rate. Fluorescence data is only recorded for intact cells and each sample should have similar event/s. If a sample has a lower number of event/s, drug treatment has likely caused lysis of the cells and fluorescence data is inaccurate as it only records cells in the population that did not lyse. In this scenario, an earlier time point such as 0.5 h rather than 1 h (prior to lysis) should be analyzed. Plating serial dilutions of your time point(s) tested will confirm whether a significant amount of lysis (i.e., cell death) occurred.

References

1. Kourtis A, Hatfield K, Baggs J et al (2019) Vital signs: Epidemiology and recent trends in methicillin-resistant and in methicillin-susceptible *Staphylococcus aureus* bloodstream infections—United States. MMWR Morb Mortal Wkly Rep 68:214–219
2. Sakoulas G, Moise-Broder PA, Schentag J et al (2004) Relationship of MIC and bactericidal

activity to efficacy of vancomycin for treatment of methicillin-resistant *Staphylococcus aureus* bacteremia. J Clin Microbiol 42:2398–2402

3. Walraven CJ, North MS, Marr-Lyon L et al (2011) Site of infection rather than vancomycin MIC predicts vancomycin treatment failure in methicillin-resistant *Staphylococcus aureus* bacteraemia. J Antimicrob Chemother 66:2386–2392
4. Radlinski L, Conlon BP (2018) Antibiotic efficacy in the complex infection environment. Curr Opin Microbiol 42:19–24
5. Michiels JE, Van den Bergh B, Verstraeten N et al (2016) Molecular mechanisms and clinical implications of bacterial persistence. Drug Resist Updat 29:76–89
6. Conlon BP (2014) *Staphylococcus aureus* chronic and relapsing infections: evidence of a role for persister cells: an investigation of persister cells, their formation and their role in *S. aureus* disease. BioEssays 36:991–996
7. Fisher RA, Gollan B, Helaine S (2017) Persistent bacterial infections and persister cells. Nat Rev Microbiol 15:453–464
8. Balaban NQ, Helaine S, Lewis K et al (2019) Definitions and guidelines for research on antibiotic persistence. Nat Rev Microbiol 17:441–448
9. Brauner A, Fridman O, Gefen O et al (2016) Distinguishing between resistance, tolerance and persistence to antibiotic treatment. Nat Rev Microbiol 14:320–330
10. Conlon BP, Rowe SE, Gandt AB et al (2016) Persister formation in *Staphylococcus aureus* is associated with ATP depletion. Nat Microbiol 1:1–7
11. Levin-Reisman I, Ronin I, Gefen O et al (2017) Antibiotic tolerance facilitates the evolution of resistance. Science 355:826–830
12. Liu J, Gefen O, Ronin I et al (2020) Effect of tolerance on the evolution of antibiotic resistance under drug combinations. Science 367:200–204
13. Andry K, Bockrath RC (1974) Dihydrostreptomycin accumulation in *E. coli*. Nature 251:534–536
14. Davis BD (1987) Mechanism of bactericidal action of aminoglycosides. Microbiol Rev 51:341–350
15. Kohanski MA, Dwyer DJ, Collins JJ (2010) How antibiotics kill bacteria: from targets to networks. Nat Rev Microbiol 8:423–435
16. Davies J, Gorini L, Davis BD (1965) Misreading of RNA codewords induced by aminoglycoside antibiotics. Mol Pharmacol 1:93–106
17. Lechner S, Lewis K, Bertram R (2012) *Staphylococcus aureus* persisters tolerant to bactericidal antibiotics. J Mol Microbiol Biotechnol 22:235–244
18. Lobritz MA, Belenky P, Porter CB et al (2015) Antibiotic efficacy is linked to bacterial cellular respiration. Proc Natl Acad Sci U S A 112:8173–8180
19. Hess DJ, Henry-Stanley MJ, Wells CL (2014) Antibacterial synergy of glycerol monolaurate and aminoglycosides in *Staphylococcus aureus* biofilms. Antimicrob Agents Chemother 58:6970–6973
20. Kim W, Zhu W, Hendricks GL et al (2018) A new class of synthetic retinoid antibiotics effective against bacterial persisters. Nature 556:103–107
21. Yoon BK, Jackman JA, Valle-González ER et al (2018) Antibacterial free fatty acids and monoglycerides: biological activities, experimental testing, and therapeutic applications. Int J Mol Sci 19:1114
22. Radlinski L, Rowe SE, Kartchner LB et al (2017) *Pseudomonas aeruginosa* exoproducts determine antibiotic efficacy against *Staphylococcus aureus*. PLoS Biol 15:e2003981
23. Radlinski LC, Rowe SE, Brzozowski R et al (2019) Chemical induction of aminoglycoside uptake overcomes antibiotic tolerance and resistance in *Staphylococcus aureus*. Cell Chem Biol 26:1355–1364.e1354
24. Jõers A, Kaldalu N, Tenson T (2010) The frequency of persisters in *Escherichia coli* reflects the kinetics of awakening from dormancy. J Bacteriol 192:3379–3384
25. Herbert S, Ziebandt A-K, Ohlsen K et al (2010) Repair of global regulators in *Staphylococcus aureus* 8325 and comparative analysis with other clinical isolates. Infect Immun 78:2877–2889
26. Udekwu KI, Parrish N, Ankomah P et al (2009) Functional relationship between bacterial cell density and the efficacy of antibiotics. J Antimicrob Chemother 63:745–757
27. Sandoval R, Leiser J, Molitoris BA (1998) Aminoglycoside antibiotics traffic to the Golgi complex in LLC-PK1 cells. J Am Soc Nephrol 9:167

Part VI

Cellular and Animal Model Systems for Studying Persistence

Chapter 16

In Vitro Models for the Study of the Intracellular Activity of Antibiotics

Frédéric Peyrusson, Tiep K. Nguyen, Julien M. Buyck, Sandrine Lemaire, Gang Wang, Cristina Seral, Paul M. Tulkens, and Françoise Van Bambeke

Abstract

Intracellular bacteria are poorly responsive to antibiotic treatment. Pharmacological studies are thus needed to determine the antibiotics which are the most potent or effective against intracellular bacteria as well as to explore the reasons for poor bacterial responsiveness. An in vitro pharmacodynamic model is described, consisting of (1) phagocytosis of preopsonized bacteria by eukaryotic cells, (2) elimination of noninternalized bacteria with gentamicin, (3) incubation of infected cells with antibiotics, and (4) determination of surviving bacteria by viable cell counting and normalization of the counts based on sample protein content. The use of strains expressing fluorescent proteins under the control of an inducible promoter allows to follow intracellular bacterial division at the individual level and therefore to monitor bacterial persisters that do not multiply anymore.

Key words Intracellular infection, Gentamicin, Antibiotic, Phagocytosis, Opsonization, Pharmacodynamics, Efficacy, Relative potency

1 Introduction

Intracellular survival of bacteria is now recognized as a major factor associated with dissemination, persistence, or recurrence of infections [1–7]. When residing inside eukaryotic cells, bacteria are indeed protected from the host humoral immune defenses and often adopt a dormant lifestyle less responsive to antibiotic action. Studies conducted over the last 10 years suggest that these dormant bacteria may correspond to bacterial persisters [8, 9]. Moreover, in order to exert their activity against intracellular bacteria, antibiotics have to gain access to the infected compartment within the cells and to express their activity in this specific environment [10, 11]. For these reasons, intracellular activity of antibiotics is unpredictable based on the simple evaluation of their activity against extracellular bacteria in broth and of their accumulation within eukaryotic cells.

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Appropriate models need to be developed for the correct assessment of the capacity of antibiotics to act upon intracellular bacteria.

We present here an in vitro model which allows studying the pharmacodynamics of antibiotics against intracellular bacteria. This model is highly flexible, being adaptable to several bacterial species or strains [12–15] as well as to many cell types [14, 16–18]. It has been used to compare the activity of commercially available antibiotics [12, 19] and of molecules in preclinical or clinical development (most of which are now registered or in the late phases in clinical trials [14, 16, 19–26]), with the aim of predicting their potential interest for the treatment of persistent infections. In the case of *Staphylococcus aureus* infections, it has been validated vs. animal models of intracellular infection [27, 28].

2 Materials

2.1 Equipment

1. Laminar flow hood: Work is performed in a laminar flow hood in a room with biosafety level adapted to the pathogenicity of the microorganism under investigation [29].
2. CO_2 incubator.
3. Bacteriology incubator.
4. Hemocytometer.
5. Spectrophotometer.

2.2 Reagents

1. Culture medium adapted for eukaryotic cell line use: Usually RPMI-1640 or DMEM, supplemented with 10% fetal calf serum.
2. Cation-adjusted Mueller-Hinton Broth (CA-MHB) and tryptic soy agar plates (TSA) (or any other specific medium more adapted to the bacterial species investigated).
3. Sterile distilled water.
4. Sterile phosphate buffer saline (PBS): 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 1 L distilled water. Adjust to pH 7.4.
5. Human serum from healthy volunteers (for bacterial opsonization).
6. 40 mg/mL gentamicin stock solution.
7. Stock solution of the antibiotic under study.
8. Reagents (*see* **Note 1**) or kit (several kits are commercially available) for protein assay according to the Folin–Ciocalteu method, also referred to as Lowry's method [30].
9. Reagents (*see* **Note 2**) or kit for cell viability assay (e.g., trypan blue exclusion assay [31], or release of the cytosolic enzyme lactate dehydrogenase (LDH) [32]).

3 Methods

The method described is illustrated in Fig. 1.

3.1 Preparation of Bacterial Suspension and of Media

1. The day before the experiment, prepare an overnight bacterial culture in 15 mL of MHB (37 °C; agitation) to obtain a stationary-phase culture.
2. Unfreeze human serum.
3. Prewarm culture medium, sterile water, and PBS at 37 °C.

3.2 Opsonization of Bacteria

Opsonization is a process by which bacteria are marked by opsonins, which are serum proteins (like antibodies or complement proteins) bridging bacteria to the cell surface in order to favor phagocytosis (*see* **Note 3**).

1. Centrifuge the overnight culture to pellet bacteria (7 min at 3200 × *g*).
2. Resuspend in 1 mL of human serum; dilute with 9 mL of eukaryotic cell culture medium (not supplemented with fetal calf serum in this case, since human serum at a final concentration of 10% is present). Do not vortex.
3. Incubate for 30–60 min at 37 °C under gentle agitation (130 rpm) [12, 33].

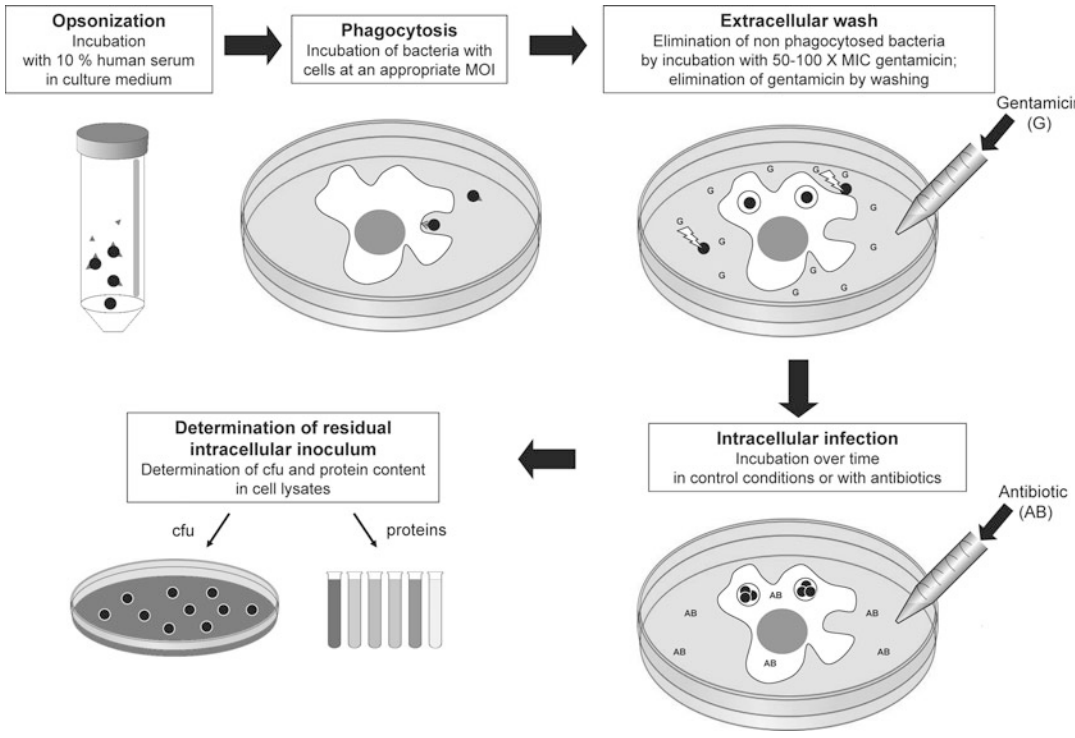

Fig. 1 In vitro model for the assessment of intracellular activity of antibiotics

3.3 Preparation of Eukaryotic Cells and Bacteria for Infection

1. If using eukaryotic cells in suspension, count them (for example using a hemocytometer) in order to obtain a density of 500,000–750,000 cells/mL (*see* **Note 4**).
2. If using adherent cells, plate them in multiwell plates. They should have reached 80% confluence at the time of the experiment. Prepare extra wells to be used for cell counting at the time of the infection.
3. Centrifuge opsonized bacteria for 7 min at 3200 × *g* and remove supernatant. Resuspend the pellet in 2 mL of PBS or culture medium and calculate the bacterial concentration, based on a calibration curve establishing the correlation between colony forming unit (CFU) counts and OD_{620nm} or on the turbidity of the bacterial suspension (McFarland).

3.4 Phagocytosis

This step is critical, in the sense that it is specific for each bacterial strain or species [12, 13, 19, 33, 34] and for the cell type to use for infection [14, 16, 17, 21] and should be adapted by the experimenter (Fig. 2). The objective is to obtain after phagocytosis an intracellular inoculum that is high enough to allow further growth of the bacteria but low enough to avoid killing the host cells (typically 10^6 CFU/mg cell protein). The general principle of this part of the protocol is explained hereafter.

1. Phagocytosis: Add bacterial suspension to cell suspension or to adherent cells in order to obtain the desired multiplicity of infection (MOI; number of bacteria/cell); when setting up the model, use in parallel different MOI (typically 1:1; 5:1; 10:1; 20:1; 50:1). Incubate at 37 °C in a CO_2 incubator for appropriate times; when setting up the model, compare different incubation times (typically 0.5 h, 1 h, 2 h).
2. Eliminate nonphagocytized bacteria either by centrifugation (cells in suspension; 7 min at 340 × *g*) or by elimination of the medium (adherent cells).
3. Reincubate infected cells during 45–60 min (37 °C; CO_2 incubator) in cell culture medium (without serum) containing gentamicin at high concentration (typically 50–100 times the minimal inhibitory concentration (MIC) for the bacterial strain used [12, 19]) in order to eliminate nonphagocytized bacteria that may adhere to the cell surface (*see* **Note 5**).
4. Wash three times with PBS at room temperature to eliminate bacterial debris and gentamicin.
5. Collect infected cells in 1 mL of sterile water in order to lyse them and allow for release of phagocytized bacteria.

6. Prepare logarithmic dilutions of the cell lysates in PBS and plate 50 µL on TSA or any other appropriate agar plate; proceed to colony counting after 24 h incubation.
7. In parallel, determine protein content of the cell lysates by the Folin–Ciocalteu method [30], using a commercial kit or the method described in **Note 1**.
8. Express the data as CFU/mg of cell protein and select for further experiments the conditions for which you obtain approximately 10^6 CFU/mg cell protein (*see* **Note 6**).

3.5 Intracellular Growth

1. Reincubate the infected cells in cell culture medium supplemented with 10% fetal calf serum. For control conditions, add gentamicin at a concentration close to the MIC (as measured in the culture medium used for the experiment) to avoid extracellular growth (Fig. 2) and, in case of cell killing, release of a small

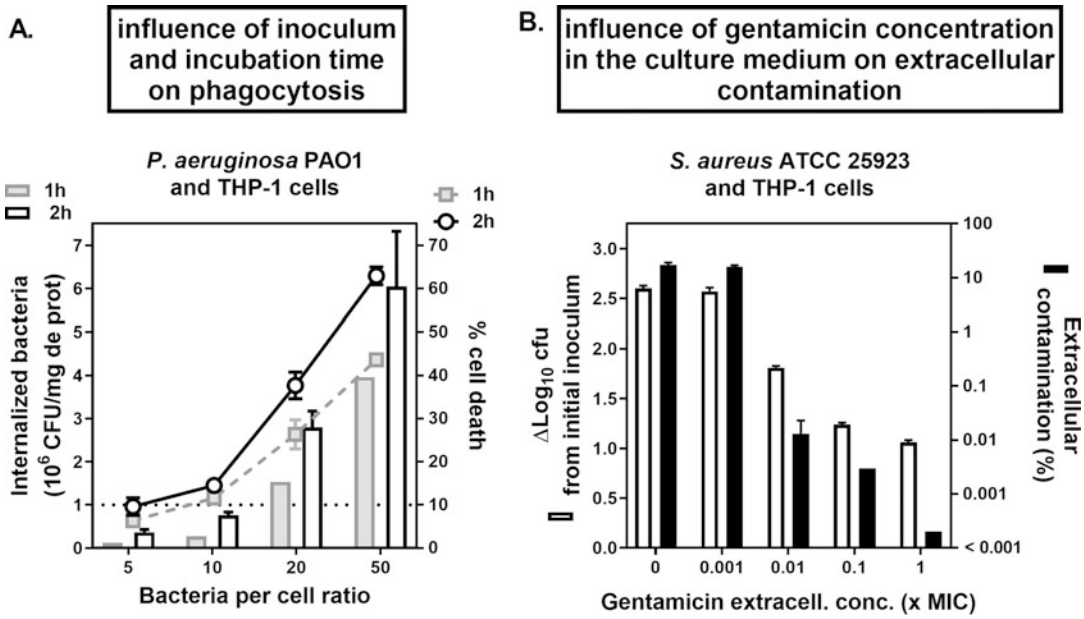

Fig. 2 Setting up a model of intracellular infection. (**a**) Determination of the optimal bacterial inoculum and phagocytosis time, as exemplified for *P. aeruginosa* PAO1 (adapted from [12]). Cells were incubated for 1 or 2 h with PAO1 at increasing bacteria-to-cell ratios (left axis). The percentage of mortality of THP-1 cells was assessed at the end of the phagocytosis period (right axis). Data for 1 h: gray symbols and bars; data for 2 h: open symbols and bars; the back bar and black dot correspond to the conditions considered as optimal for this model (dotted line: 10^6 CFU/mg protein with $<10\%$ cell toxicity). (**b**) Determination of the optimal concentration of gentamicin to add to culture medium of controls during incubation to avoid extracellular contamination, as exemplified for *S. aureus* ATCC25923 (adapted from [19]). Left axis: change in intracellular inoculum (log scale) after 24 h of incubation of infected cells in the presence of increasing concentrations of gentamicin (expressed in multiples of the MIC. Right axis: percentage of contamination of the extracellular medium in these conditions as assessed by the counting of colonies after plating of pooled culture fluids and washing media (limit of detection: 0.001%)

number of bacteria into the medium [19]. For experimental conditions, add the antibiotic you wish to test at the appropriate concentration in the culture medium (*see* **Notes 7** and **8**).

2. At the end of the incubation period, wash the cells three times in PBS and collect them in sterile distilled water as explained above (Subheading 3.4). Proceed to plating, CFU counting and protein assay.

3.6 Assessment of Antibiotic Intracellular Activity

The model described here allows to monitor antibiotic activity against intracellular bacteria over time or as a function of the extracellular concentration of the antibiotic (Fig. 3) [12, 19].

1. Considering time effects, bacterial growth is often delayed inside the cells (lag phase of a few hours), so that bacterial killing by antibiotics occurs slower than in broth. Moreover, the rate of bacterial killing by antibiotics is often biphasic, with

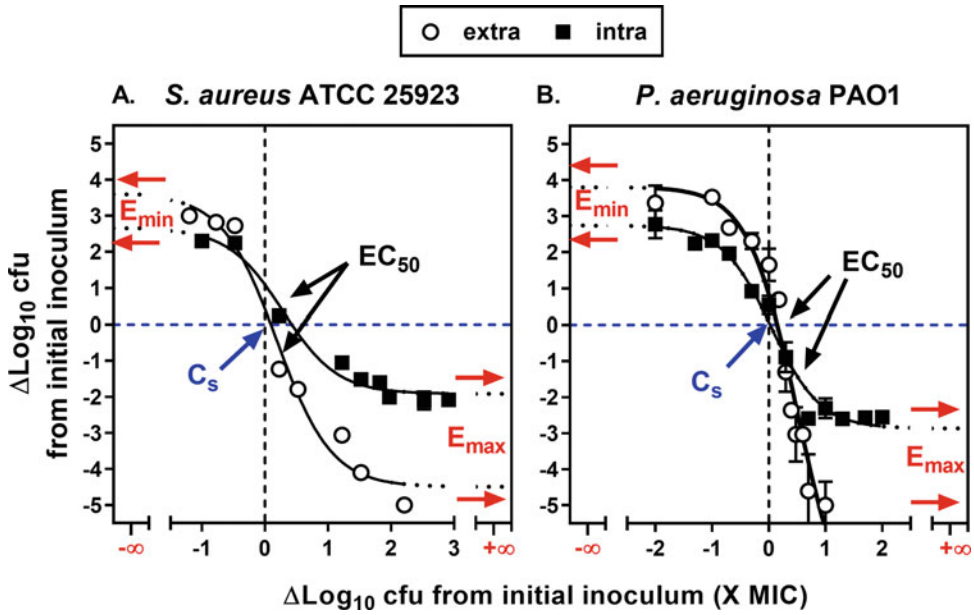

Fig. 3 Concentration–effect relationship for the extracellular and intracellular activity of antibiotics, exemplified for moxifloxacin against *S. aureus* (**a**) and *P. aeruginosa* (**b**). Comparison of the activity of moxifloxacin after 24 h incubation with moxifloxacin in broth (extracellular activity; open symbols) or in infected THP-1 cells (closed symbols). The ordinate shows the change in the number of CFU per mL (extracellular) or per mg cell protein (intracellular) compared to the postphagocytosis inoculum (blue horizontal line at 0). The abscissa shows the antibiotic concentration expressed as the $\log_{10}$ of its MIC in broth. The dotted line shows the MIC value. Data are used to fit Hill equations (slope factor = 1) and derive the pertinent key pharmacodynamic parameters, namely (1) E_{min} (change in CFU for an infinitely low antibiotic concentration; in red); (2) E_{max} (relative efficacy; maximal reduction in inoculum as extrapolated for an infinitely large concentration, in $\log_{10}$ CFU units compared to the original inoculum; in red); (3) EC_{50} (relative potency; concentration causing a reduction of the inoculum halfway between E_{min} and E_{max}, in black); (4) C_s (static concentration; concentration resulting in no apparent bacterial growth; in blue). Constructed based on data presented in [12, 15]

a first rapid drop in the number of CFUs, followed by a slower killing, which can even correspond to a plateau (no further decrease in CFUs). This is one of the hallmarks of persisters (Fig. 4).

2. Considering concentration effects, performing experiments with broad ranges of extracellular concentrations (from sub-MIC values to many times the MIC) allows obtaining full concentration–response curves for fitting with sigmoid regressions (Fig. 3).
3. Using the corresponding Hill's equation, key pharmacological descriptors of activity can be calculated.
   (a) The relative minimal efficacy (E_{min}; in $\log_{10}$ CFU units), that is, the increase in the number of CFU for an infinitely low antibiotic concentration compared to the original postphagocytosis inoculum.
   (b) The relative maximal efficacy (E_{max}; in $\log_{10}$ CFU units), that is, the decrease in the number of CFU for an infinitely large concentration of antibiotic.
   (c) The relative potency (EC_{50}; in mg/L or in multiples of MIC), that is, the concentration of antibiotic yielding a response half-way between E_{min} and E_{max}.
   (d) The static concentration (C_s; in mg/L or in multiple of MIC), that is, the concentration of antibiotic resulting in no apparent bacterial growth compared to the original inoculum [15].
4. Three major observations have been made with this type of model (Figs. 3 and 4).
   (a) First, the relative minimal efficacy is in general similar in the extracellular and intracellular models for facultative intracellular bacteria. Intracellular E_{min} should be considered as an "apparent" intracellular value, because in this case, the presence of extracellular bacteria that are not killed in the medium by subinhibitory concentrations of antibiotic cannot be excluded.
   (b) Second, the static concentration against intracellular bacteria (i.e., the antibiotic concentration preventing bacterial growth) is in most cases close to the MIC, suggesting that the potency of the drug is not directly correlated with its accumulation inside the cells, possibly because of poor intracellular bioavailability. The molecular reasons for this loss of potency inside the cells still remain to be established.
   (c) Third, the antibiotic maximal efficacy is in most cases much lower against intracellular bacteria than against extracellular bacteria, suggesting poor bacterial

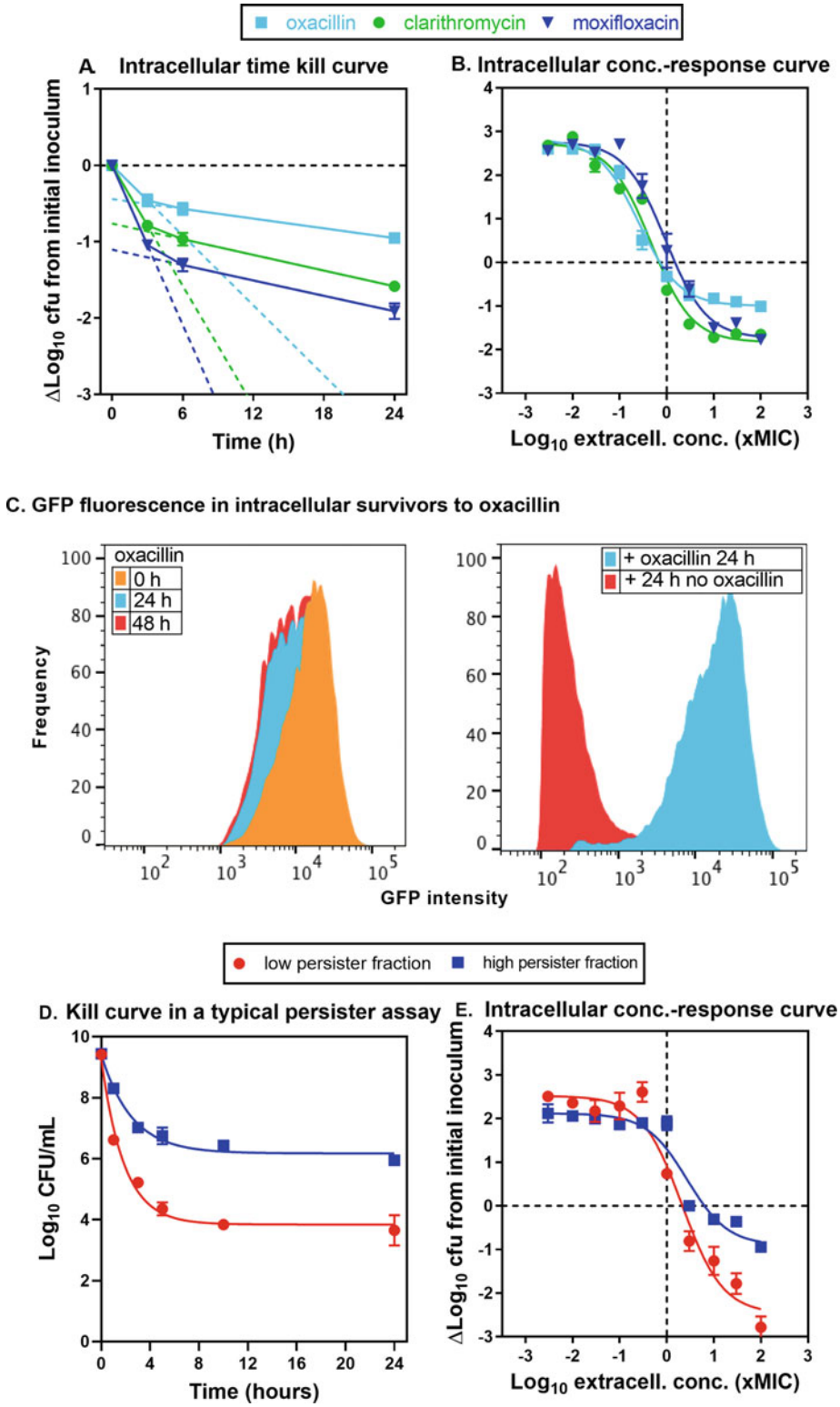

Fig. 4 Demonstration of intracellular persisters in *S. aureus* upon exposure to antibiotics at high concentrations. (**a**) Time-kill curves of *S. aureus* SH1000 in J774 mouse macrophages incubated with oxacillin,

responsiveness to antibiotic action in the intracellular environment. By means of a fluorescence dilution technique (described in Chapter 18), this has been recently ascribed for *S. aureus* to the fact that intracellular survivors have adopted a persister phenotype, characterized by a nondividing state, and reversible in permissive cells as soon as the antibiotic pressure is relieved [18]. The E_{max} value differs from one antibiotic to the other against a same strain, but may differ from one strain to the other with a same antibiotic. These discrepancies could find their explanation in the capacity of different antibiotics or strains to generate persisters [35]. Again the reasons for these differences need to be established.

4 Notes

1. Protein assay can be performed without any commercial kit, using the protocol described by Lowry [30]. Reagents required are Biuret reagent (extemporaneous mixture of 100 mL 2% Na_2CO_3, 1 mL 2% potassium sodium tartrate, 1 mL 1% $CuSO_4.5H_2O$), 2 N Folin–Ciocalteu reagent (diluted to 1 N), 1 N NaOH, and a standard (100 μg/mL bovine albumin). In brief, incubate 0.5 mL of cell lysate (or dilution thereof), blank (medium in which cells were collected), water (solvent of albumin standard) or albumin standard during 30–120 min with 0.5 mL 1 N NaOH. Subsequently, add

Fig. 4 (continued) clarithromycin, or moxifloxacin at 50× their respective MIC. The graph shows the biphasic killing rate, with a fast killing during the first 3 h and a slower killing thereafter (highlighted by dotted lines). The equation of these linear relationships allows to calculate a minimum duration of killing (MDK) for 90% of the population comprised between 2.9 and 6.6 h for the first phase but longer than 24 h for the second phase (adapted from [18]). (**b**) Concentration–response curves for the same antibiotics after 24 h of incubation of infected cells. A plateau is reached corresponding to a maximal reduction of 1–1.8 $\log_{10}$ CFU from the postphagocytosis, depending on the drug (adapted from [18]). (**c**) Flow cytometric profiles of the frequency of events as a function of GFP intensity over time for samples collected from an experiment similar to that described in panel **b** and incubated 0 h (postphagocytosis), 24 h, or 48 h with 50× the MIC of oxacillin (left) or 24 h with oxacillin then reincubated for 24 h in the absence of antibiotic (right). Cells were infected by SH1000 transformed by a plasmid expressing GFP under the control of an inducible promoter. The inducer is added during the prephagocytosis cultures only. Once the bacteria have been internalized, any dilution of the fluorescence signal can be interpreted as denoting bacterial division (adapted from [18]). (**d**) Time-kill curve of extracellular bacteria in stationary cultures exposed to moxifloxacin at 100× its MIC to calculate their persister fraction. The graph compares two clinical isolates harboring low (red) and high (blue) persister fractions (adapted from [35]). (**e**) Concentration–response curves for the same isolates in an intracellular model of infected THP-1 human monocytes incubated during 24 h with moxifloxacin. The graph shows that the E_{max} of moxifloxacin is higher (more negative) for the isolate harboring the lower persister fraction in stationary-phase culture (adapted from [35])

5 mL of Biuret reagent and incubate for 10–20 min. Next, add 0.5 ml of 1 N Folin reagent to each tube and read absorbance at 660 nm after 30 min of incubation (the last step needs to be done tube by tube and with a timer; incubation time should be strictly the same for each tube). The concentration of proteins in the sample is then calculated as ($[OD_{sample} - OD_{blank}]/[OD_{standard} - DO_{water}]$) × 100 µg/mL [standard concentration] × dilution factor) [3].

2. Viability can be easily assessed using a trypan blue exclusion test (vital colorant excluded from viable cells). To this effect, add 100 µL of cell suspension to 900 µL of trypan blue reagent, incubate for 10 min at 37 °C and determine the proportion of dead cells (colored in blue) by cell counting using a haemocytometer. An alternative method consists of measuring the release of LDH, a cytosolic enzyme, in the supernatant of a cell culture, which occurs upon permeabilization of the cell membrane. LDH viability kits are commercially available. The assay can also be performed using the method of Vassault [32], which measures the consumption of NADH upon reduction of pyruvate in lactate by LDH (Fig. 5).

   In brief, mix 50 µL of culture medium or 10 µL of cell lysate with 2.5 mL of 0.244 mM NADH solution in Tris buffer (81.3 mM Tris, 203.3 mM NaCl). Add 500 µL of 9.76 mM natrium pyruvate (prepared in the same buffer) and follow NADH consumption by measuring optical density at 339 nm immediately and then every min during 5 min. Cell mortality is evaluated by the ratio between LDH activity in the supernatant (estimated by $[OD_{0\ min} - OD_{5\ min}]$/µL of medium × total volume of the culture medium) and the total activity in the culture (sum of total activity in supernatant and total activity in cell lysate estimated as ($[OD_{0\ min} - OD_{5\ min}]$/µL of medium × total volume of cell lysate)).

3. When using obligatory or facultative intracellular organisms which are specifically equipped to use the serum complement to increase phagocytosis, opsonization causes massive infection of the cells [36]. Preopsonization is therefore not systematically required [37] and, alternatively, culture medium could be supplemented with decomplemented serum or calf serum (heated for 30 min at 56 °C [38]) to reduce phagocytosis in order to reach postphagocytosis inocula compatible with maintenance of cell viability for 24 h.

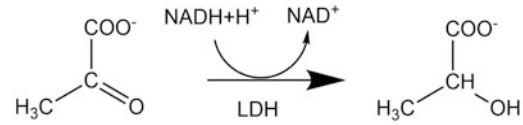

Fig. 5 Conversion of pyruvate to lactate by lactate dehydrogenase (LDH)

4. The number of eukaryotic cells to use depends on the virulence of the bacterial strain. For cytotoxic bacterial strains or species, use a higher eukaryotic cell number in order to keep enough cells after phagocytosis, as some killing may occur during this step [12].
5. A limitation of this assay is that the strain has to be susceptible to gentamicin. This antibiotic is selected for the elimination of nonphagocytized bacteria because it is rapidly bactericidal while at the same time entering only very slowly inside eukaryotic cells. It is therefore important to test for the susceptibility of the bacterial strain to gentamicin (MIC determination) before starting the experiment. Use of lysostaphin as a lytic agent for some extracellular bacteria (*S. aureus*) is also proposed in the literature but we showed that it enters inside the cells and may thus affect intracellular viability [33].
6. Depending on the virulence of the strain and its capacity to multiply intracellularly, it is important to check in parallel for the viability of the cells at the end of the phagocytosis period as well as at the end of the experiment. To this effect, a viability assay (trypan blue exclusion assay or LDH release assay, *see* **Note 2**) should be run in parallel and the postphagocytosis inoculum should be selected so as to guarantee cell viability.
7. Antibiotics or antibacterial agents (or even their solvent if not soluble in water) may also be toxic to eukaryotic cells. Again, it is important to check for cell viability in the presence of the tested agent for correct interpretation of the data. Massive cell death induced by the antibacterial agent can trigger bacterial release into the culture medium and therefore lead to the evaluation of the activity of the tested agent against extracellular bacteria rather than against intracellular bacteria [39].
8. For highly bactericidal antibiotics, check that the amount of carried-over antibiotic does not impair bacterial growth on the plates [33]. This can be done by comparing the number of CFU on plates from lysates preexposed or not to 12.5 mg/L charcoal (adsorbing residual antibiotic) during 10 min [20] or by plating bacteria on agar supplemented with 0.4% charcoal [13].

Acknowledgments

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References

1. Garzoni C, Kelley WL (2011) Return of the Trojan horse: intracellular phenotype switching and immune evasion by *Staphylococcus aureus*. EMBO Mol Med 3:115–117
2. Anderson GG, Martin SM, Hultgren SJ (2004) Host subversion by formation of intracellular bacterial communities in the urinary tract. Microbes Infect 6:1094–1101
3. Cossart P, Sansonetti PJ (2004) Bacterial invasion: the paradigms of enteroinvasive pathogens. Science 304:242–248
4. Mehlitz A, Rudel T (2013) Modulation of host signaling and cellular responses by *Chlamydia*. Cell Commun Signal 11:90
5. Rohde M, Chhatwal GS (2013) Adherence and invasion of streptococci to eukaryotic cells and their role in disease pathogenesis. Curr Top Microbiol Immunol 368:83–110
6. Alder KD, Lee I, Munger AM, Kwon HK et al (2020) Intracellular *Staphylococcus aureus* in bone and joint infections: a mechanism of disease recurrence, inflammation, and bone and cartilage destruction. Bone 141:115568
7. Del Mar CM, Torrents E (2020) Differential adaptability between reference strains and clinical isolates of *Pseudomonas aeruginosa* into the lung epithelium intracellular lifestyle. Virulence 11:862–876
8. Adams KN, Takaki K, Connolly LE et al (2011) Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. Cell 145:39–53
9. Helaine S, Cheverton AM, Watson KG et al (2014) Internalization of *Salmonella* by macrophages induces formation of nonreplicating persisters. Science 343:204–208
10. Carryn S, Chanteux H, Seral C et al (2003) Intracellular pharmacodynamics of antibiotics. Infect Dis Clin N Am 17:615–634
11. Van Bambeke F, Barcia-Macay M, Lemaire S, Tulkens PM (2006) Cellular pharmacodynamics and pharmacokinetics of antibiotics: current views and perspectives. Curr Opin Drug Discov Devel 9:218–230
12. Buyck JM, Tulkens PM, Van Bambeke F (2013) Pharmacodynamic evaluation of the intracellular activity of antibiotics towards *Pseudomonas aeruginosa* PAO1 in a model of THP-1 human monocytes. Antimicrob Agents Chemother 57:2310–2318
13. Chalhoub H, Harding SV, Tulkens PM, Van Bambeke F (2019) Influence of pH on the activity of finafloxacin against extracellular and intracellular *Burkholderia thailandensis*, *Yersinia pseudotuberculosis* and *Francisella philomiragia* and on its cellular pharmacokinetics in THP-1 monocytes. Clin Microbiol Infect 26:1254.e1–1254.e8
14. Lemaire S, Kosowska-Shick K, Appelbaum PC et al (2010) Cellular pharmacodynamics of the novel biaryloxazolidinone radezolid: studies with infected phagocytic and nonphagocytic cells, using *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Listeria monocytogenes*, and *Legionella pneumophila*. Antimicrob Agents Chemother 54:2549–2559
15. Lemaire S, Kosowska-Shick K, Appelbaum PC et al (2011) Activity of moxifloxacin against intracellular community-acquired methicillin-resistant *Staphylococcus aureus*: comparison with clindamycin, linezolid and co-trimoxazole and attempt at defining an intracellular susceptibility breakpoint. J Antimicrob Chemother 66:596–607
16. Lemaire S, Glupczynski Y, Duval V et al (2009) Activities of ceftobiprole and other cephalosporins against extracellular and intracellular (THP-1 macrophages and keratinocytes) forms of methicillin-susceptible and methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 53:2289–2297
17. Lemaire S, Olivier A, Van Bambeke F et al (2008) Restoration of susceptibility of intracellular methicillin-resistant *Staphylococcus aureus* to beta-lactams: comparison of strains, cells, and antibiotics. Antimicrob Agents Chemother 52:2797–2805
18. Peyrusson F, Varet H, Nguyen TK et al (2020) Intracellular *Staphylococcus aureus* persisters upon antibiotic exposure. Nat Commun 11:2200
19. Barcia-Macay M, Seral C, Mingeot-Leclercq MP et al (2006) Pharmacodynamic evaluation of the intracellular activities of antibiotics against *Staphylococcus aureus* in a model of THP-1 macrophages. Antimicrob Agents Chemother 50:841–851
20. Lemaire S, Tulkens PM, Van Bambeke F (2011) Contrasting effects of acidic pH on the extracellular and intracellular activities of the anti-gram-positive fluoroquinolones moxifloxacin and delafloxacin against *Staphylococcus aureus*. Antimicrob Agents Chemother 55:649–658
21. Lemaire S, Van Bambeke F, Appelbaum PC, Tulkens PM (2009) Cellular pharmacokinetics and intracellular activity of torezolid (TR-700): studies with human macrophage (THP-1) and endothelial (HUVEC) cell lines. J Antimicrob Chemother 64:1035–1043

22. Lemaire S, Van Bambeke F, Tulkens PM (2009) Cellular accumulation and pharmacodynamic evaluation of the intracellular activity of CEM-101, a novel fluoroketolide, against *Staphylococcus aureus*, *Listeria monocytogenes*, and *Legionella pneumophila* in human THP-1 macrophages. Antimicrob Agents Chemother 53:3734–3743
23. Melard A, Garcia LG, Das D et al (2013) Activity of ceftaroline against extracellular (broth) and intracellular (THP-1 monocytes) forms of methicillin-resistant *Staphylococcus aureus*: comparison with vancomycin, linezolid and daptomycin. J Antimicrob Chemother 68:648–658
24. Peyrusson F, Butler D, Tulkens PM, Van Bambeke F (2015) Cellular pharmacokinetics and intracellular activity of the novel peptide deformylase inhibitor GSK1322322 against *Staphylococcus aureus* laboratory and clinical strains with various resistance phenotypes: studies with human THP-1 monocytes and J774 murine macrophages. Antimicrob Agents Chemother 59:5747–5760
25. Peyrusson F, Tulkens PM, Van Bambeke F (2018) Cellular pharmacokinetics and intracellular activity of gepotidacin against *Staphylococcus aureus* isolates with different resistance phenotypes in models of cultured phagocytic cells. Antimicrob Agents Chemother 62: e02245–e02217
26. Peyrusson F, Van Wessem A, Dieppois G et al (2020) Cellular pharmacokinetics and intracellular activity of the bacterial fatty acid synthesis inhibitor, afabicin desphosphono against different resistance phenotypes of *Staphylococcus aureus* in models of cultured phagocytic cells. Int J Antimicrob Agents 55:105848
27. Sandberg A, Jensen KS, Baudoux P et al (2010) Intra- and extracellular activities of dicloxacillin against *Staphylococcus aureus in vivo* and *in vitro*. Antimicrob Agents Chemother 54:2391–2400
28. Sandberg A, Jensen KS, Baudoux P et al (2010) Intra- and extracellular activity of linezolid against *Staphylococcus aureus in vivo* and *in vitro*. J Antimicrob Chemother 65:962–973
29. U.S. Department of Health and Human Services (2009) Biosafety in microbiological and biomedical laboratories, vol 5. Available via https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2009-P.pdf. Accessed 6 Oct 2020
30. Lowry OH, Rosebrough AL, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
31. Strober W (2001) Trypan blue exclusion test of cell viability. Curr Protoc Immunol Appendix 3:Appendix 3B
32. Vassault A (1987) Lactate dehydrogenase. In: Bergemeyer HU (ed) Methods in enzymatic analysis, vol III: enzyme I oxydoreductases, transferases. VHC Publishers, Veinheim
33. Seral C, Van Bambeke F, Tulkens PM (2003) Quantitative analysis of gentamicin, azithromycin, telithromycin, ciprofloxacin, moxifloxacin, and oritavancin (LY333328) activities against intracellular *Staphylococcus aureus* in mouse J774 macrophages. Antimicrob Agents Chemother 47:2283–2292
34. Garcia LG, Lemaire S, Kahl BC et al (2012) Influence of the protein kinase C activator phorbol myristate acetate on the intracellular activity of antibiotics against hemin- and menadione-auxotrophic small-colony variant mutants of *Staphylococcus aureus* and their wild-type parental strain in human THP-1 cells. Antimicrob Agents Chemother 56:6166–6174
35. Nguyen TK, Peyrusson F, Dodémont M et al (2020) The persister character of clinical isolates of *Staphylococcus aureus* contributes to faster evolution to resistance and higher survival in THP-1 monocytes: a study with moxifloxacin. Front Microbiol 11:587364
36. Drevets DA, Campbell PA (1991) Roles of complement and complement receptor type 3 in phagocytosis of *Listeria monocytogenes* by inflammatory mouse peritoneal macrophages. Infect Immun 59:2645–2652
37. Seral C, Carryn S, Tulkens PM, Van Bambeke F (2003) Influence of P-glycoprotein and MRP efflux pump inhibitors on the intracellular activity of azithromycin and ciprofloxacin in macrophages infected by *Listeria monocytogenes* or *Staphylococcus aureus*. J Antimicrob Chemother 51:1167–1173
38. Carryn S, Van Bambeke F, Mingeot-Leclercq MP, Tulkens PM (2002) Comparative intracellular (THP-1 macrophage) and extracellular activities of beta-lactams, azithromycin, gentamicin, and fluoroquinolones against *Listeria monocytogenes* at clinically relevant concentrations. Antimicrob Agents Chemother 46:2095–2103
39. Lemaire S, Bogdanovitch T, Chavez-Bueno S et al (2006) Bactericidal activity of ceragenin CSA-13 against intracellular MSSA, hospital-acquired (HA) and Community-acquired (CA) MRSA, and VISA in THP-1 macrophages: relation to cellular toxicity ? Poster presented at the 46th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, USA, 27–30 Sept 2006

Chapter 17

Analysis of *Salmonella* Persister Population Sizes, Dynamics of Gut Luminal Seeding, and Plasmid Transfer in Mouse Models of Salmonellosis

Erik Bakkeren, Joshua P. M. Newson, and Wolf-Dietrich Hardt

Abstract

A previously unappreciated link between persisters and the emergence and spread of antibiotic resistance has been recently established. The bulk of this research has been conducted in vitro, but some studies are beginning to elucidate the importance of persister reservoirs in both antibiotic treatment failure and the spread of antibiotic resistance using in vivo models. In order to further this research, careful analyses of the mechanisms of persister reservoir formation as well as the dynamics of persister survival and postantibiotic regrowth are of importance. Here, we present a mouse model to quantitatively study *Salmonella* persisters in vivo. By using neutral unique sequence barcodes, we describe the quantitative analysis of rare events (aka bottlenecks) associated with persister reservoir formation, survival, and reseeding of the gut lumen. This provides quantitative data for persister-fueled plasmid transfer in vivo. Although this chapter describes analysis of *Salmonella* persisters in a mouse model, these concepts can be applied to any experimental system provided that tractable experimental systems are present.

Keywords Persistence, Mouse model, *Salmonella* Typhimurium, Ceftriaxone, Plasmid transfer, Antibiotic resistance plasmids, ESBL, Pathogen evolution

1 Introduction

The intestine of humans and animals provides a large reservoir where bacteria can survive, grow, and interact. In these niches, densities of Enterobacteriaceae can reach up to 10^9 CFU per gram of feces [1]. This can support efficient horizontal gene transfer (HGT) of fitness or virulence determinants [2–5], including plasmids harboring antibiotic resistance genes [6–8]. Moreover, invasive enteropathogens such as *Salmonella enterica* serovar Typhimurium (*S*.Tm) can also colonize the gut environment,

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leading to inflammation-mediated blooms of Enterobacteriaceae [9–11] that can further increase HGT [3]. In the case of *S*.Tm, invasion is required to trigger strong inflammation and enhance transmission [12]. As *S*.Tm evolved to become a better invasive pathogen and trigger more inflammation (due to the acquisition of SPI-1 and SPI-2), it was also able to coincidentally survive within host tissues [5]. This allowed *S*.Tm to survive inside of immune cells that favor the formation of persisters [13].

An increasing number of studies have investigated persisters in vivo, allowing for important new insights into persister biology that cannot be achieved by solely in vitro experimentation [7, 14–18]. Recently, we established a link between S.Tm persister formation and antibiotic resistance plasmid transfer among different Enterobacteriaceae [7]. S.Tm cells that harbor plasmids can invade into host tissues, survive antibiotic therapy as persisters within tissue reservoirs, and thereby form long-term reservoirs for plasmid transfer. Later, such plasmid-bearing bacterial cells can reseed the gut lumen to transfer plasmids to other strains of *S*.Tm or *Escherichia coli* that colonize the gut lumen [7]. Our work showed that studying persisters in vivo is not only important for understanding why antibiotic treatment failure can be common, but also that persisters may have important evolutionary consequences, facilitating the spread of genes on mobile genetic elements [5].

In this chapter, we outline methods to study *Salmonella* persisters in vivo in a quantitative fashion, and investigate their influence on plasmid spread in the gut lumen. We specifically describe how to investigate persister populations in both typhoidal and nontyphoidal models for salmonellosis in mice. We will cover reservoir formation in vivo, tracking the postantibiotic treatment seeding of persisters back into the gut lumen using population dynamic approaches, and monitoring the efficiency of plasmid transfer after gut lumen seeding.

Since this entire process is dynamic and subject to several population bottlenecks, we have devised a population dynamics approach. Specifically, we use wild-type isogenic tagged strains (WITS), which contain a 40 bp genetic barcode (or 'tag') coupled to an antibiotic resistance marker that can be detected by qPCR [19]. This has advantages over simple endpoint analysis, since it can capture dynamic processes or rare events that would not be resolved by only counting CFUs at the end of the experiment. Within-host pressures on bacterial populations, such as killing or migration, exert a bottleneck effect on the population of tagged bacteria. Analysis of the bottleneck effect can also be influenced by changing the experimental design, for example by introducing more tags or by diluting the tagged strains in an untagged isogenic strain. Thus, depending on the size of the expected bottleneck, WITS-carrying strains can be introduced at equal ratios, or diluted evenly in excess

of an untagged isogenic strain. By performing qPCR and observing the relative abundance of the different tags (i.e., their evenness), the size of the bottleneck can be estimated. In short, the narrower the bottleneck, the more unevenness at lower dilution. These differences cannot be detected by CFU enumeration, as rapid growth after a bottleneck can compensate for expected population size decreases. However, the relative distribution of the different tags will remain "imprinted" in this growing population and can therefore be read out at any time later. This strategy has been used in previous work [19–23]. Another advantage to the method presented here is that we do not ignore the role of interactions with other bacteria. Since WITS tags can be introduced onto conjugative plasmids, we can also study the plasmid transfer dynamics between different strains by looking at the diversity of WITS tags in the donors, the plasmids and/or the recipients that have obtained a plasmid (i.e., transconjugants). Using this approach, we have shown that plasmid transfer from persisters reseeding the gut lumen from tissue reservoirs is a rare event. These rare events are followed by rapid spread of the plasmid within a recipient population and this spread is mainly driven by transconjugant-to-recipient spread [7]. It is important to reemphasize that all of these observations are dependent on the dilution of WITS tags used within an untagged strain. In simple cases, a small number of different WITS tags and enumeration by qPCR can suffice. In this chapter, we describe analysis using just seven WITS tags. However, to capture an entire multistep process, an increased number of unique barcode-tagged strains that can be identified with deep sequencing may be of advantage; for example, we have shown in recent experiments that more than 2000 tags can be introduced to capture numerous aspects of gut luminal pathogen growth in one experiment [24].

To illustrate the approach, we focus on two mouse models for salmonellosis, which have been used to study persisters and their evolutionary consequences in vivo. The two models are each useful to address slightly different questions, and can yield complementary insights. The intraperitoneal or intravenous infection model is advantageous in that it bypasses the need for gut luminal colonization in order to form persister reservoirs within host tissues. This allows the gut luminal microbiota to remain more intact, and ensures that no persisters can be formed in the gut lumen, which might otherwise lead to interactions with the second, recipient strain introduced at a later time. The localization of persister reservoirs is also different from the peroral infection model (*see* below). Upon intraperitoneal or intravenous infection, persisters form primarily in the spleen, liver, and gall bladder (i.e., upon systemic infection). This mimics a typhoidal *Salmonella* infection, where *S.* Tm can persist in the gall bladder for long periods of time

[25]. Additionally, we describe how the experimental protocol can be modified for an oral infection model that recapitulates the normal fecal–oral route of transmission of nontyphoidal salmonellosis, leading to diarrheal disease. In this model, large numbers of persisters are formed in the intestinal mucosa and the gut associated lymphatic tissue, including the gut draining mesenteric lymph nodes. In both models, since persister reservoirs can be differentiated from transconjugants, the formation of new reservoirs after plasmid transfer can also be observed [7, 14–16]. This can inform about the dynamic nature of persister reservoirs and their interaction with gut luminal plasmid-bearing microbial populations.

Although this chapter exclusively describes *Salmonella* persisters in a murine model, using antibiotic resistance plasmid exchange as a measure for interactions between persister bacteria and recipients can be used in many different experimental systems. For example, within the gut, the transfer of mobile genetic elements is not limited to antibiotic resistance plasmids. It would be important to understand to what extent persisters can fuel the transfer of virulence-encoding determinants, for example carried on plasmids or bacteriophages. Additionally, several different environments can also allow the formation of long-term reservoirs of persisters that can interact with different types of bacteria over time. This could include chronic infections by other pathogens (e.g., polymicrobial urinary tract infections of *E. coli*), biofilms, or in sewage tanks.

2 Materials

1. LB medium: Add 10 g tryptone, 5 g yeast extract, and 10 g NaCl into a 1 L Schott bottle. Add distilled water to yield a total volume of 1 L. Mix well to fully dissolve, then autoclave at 121 °C for 20 min.
2. MacConkey agar plates: Add the required amount of powder (according to the manufacturer's instructions) into a 1 L Schott bottle. Add distilled water to yield a total volume of 1 L and mix well to fully suspend the powder. Autoclave at 121 °C for 20 min and allow the media to cool before adding antibiotics. Add either 50 μg/mL streptomycin, 50 μg/mL kanamycin, or 15 μg/mL chloramphenicol, as required. Pour approximately 20 mL into each petri dish.
3. Phosphate-buffered saline (PBS): Pour 800 mL of distilled water into a 1 L Schott bottle. Add 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4. Adjust to pH 7.4 with HCl, then add distilled water to a total volume of 1 L. Autoclave at 121 °C for 20 min to sterilize.

4. Antibiotics: Dissolve 0.3 g/mL of ciprofloxacin; 0.5 g/mL of streptomycin; 50 mg/mL of streptomycin; or 50 mg/mL of kanamycin in distilled water. Dissolve 15 mg/mL of chloramphenicol in ethanol. Dissolve 0.15 g/mL of ceftriaxone in PBS. Prepare 2 g/L of ampicillin in tap water for drinking water. Vortex solutions to completely dissolve, then filter sterilize through a 0.22 μm membrane filter.
5. Mice: Experiments can be performed in specific pathogen-free C57BL/6 J or 129S6/SvEvTac mice, as required. Other lines of interest or knockout mice can also be used. Before the experiment, collect a fecal pellet, suspend it in sterile PBS and plate it on MacConkey agar (without antibiotics) to verify the absence of *E. coli* (red colonies) or other Enterobacteriaceae which may interfere with the infection (or the interpretation of the results).
6. Bacterial strains: *Salmonella* Typhimurium strains engineered to carry a unique 40 nucleotide sequence and a resistance cassette (encoding either kanamycin or chloramphenicol resistance, as required). Additional WITS-tagged strains can be generated by PCR using the above strains as a DNA template (e.g., using primers described in Subheading 3.1.5). In this example, we used seven different WITS tags. P22 phage transduction can be used to transfer the WITS tag and linked resistance cassette into S.Tm mutant strains, as required.
7. Primers for qPCR analysis of WITS tags: Primers are listed in Table 1 (5′ to 3′ direction).
8. DNA purification kit (e.g., the QIAmp DNA mini kit [QIAGEN]).
9. Lambda red plasmids: pKD46 encodes the lambda red genes under an arabinose-inducible promoter [26]. If gene deletion mutants are required, inactivation of genes can be performed through lambda red using pKD3 or pKD4 as templates with cassettes encoding chloramphenicol or kanamycin resistance, respectively.
10. 37 °C incubator for incubation of plates and cultures.
11. Rotating wheel for incubation of cultures (approximately 50 rpm).
12. Steel gavage needles for orogastric administration of bacteria and antibiotics.
13. 26G (0.45 × 12 mm) injection needles for intraperitoneal administration of bacteria and antibiotics.
14. 5 mm steel balls and TissueLyser (QIAGEN) for organ homogenization.

Table 1
qPCR primers used in this chapter

Primer name	Sequence (5′ to 3′)
WITS1 FW	ACG ACA CCA CTC CAC ACC TA
WITS2 FW	ACC CGC AAT ACC AAC TC
WITS11 FW	ATC CCA CAC ACT CGA TCT CA
WITS13 FW	GCT AAA GAC ACC CCT CAC TCA
WITS17 FW	TCA CCA GCC CAC CCC CTC A
WITS19 FW	GCA CTA TCC AGC CCC ATA AC
WITS21 FW	ACA ACC GAT CAC TCT CC
ydgA RV	GGC TGT CCG CAA TGG GTC

3 Methods

3.1 Identification of Adequate Donor–Recipient Pairs

3.1.1 Determining the MIC of Relevant Strains to Antibiotics Used in the Mouse Model

1. Grow donors and recipients overnight at 37 °C in LB, with antibiotics as appropriate (to prevent plasmid loss by the donor strain and to minimize the risk of contamination).
2. Subculture cells 1:20 in 2 mL of LB without antibiotics for 4 h at 37 °C on a rotating wheel.
3. Fill a flat-bottom transparent 96-well plate with 100 μL of LB containing twofold dilutions of all relevant antibiotics. This is achieved by filling the first well with 200 μL of LB containing the highest concentration of antibiotic (e.g., for *Salmonella* spp. 50 μg/mL for streptomycin and kanamycin; 15 μg/mL for chloramphenicol; 100 μg/mL for ampicillin; 64 μg/mL for ceftriaxone; 20 μg/mL for ciprofloxacin; 400 μg/mL for gentamycin). Add 100 μL of LB into all remaining wells and serially transfer 100 μL to achieve twofold dilutions. Be sure to include a no-antibiotic growth control (*see* **Note 1**).
4. Dilute subcultures in PBS and add 10 μL of 10^{-3}-diluted cells to each well (total seeded cell density of 10^5 cells/mL). Be sure to include a no-bacteria sterility and background subtraction control.
5. Incubate plates for 16 h at 37 °C and 120 rpm to allow bacterial growth. Measure the $OD600_{nm}$ using a spectrophotometer. Subtract the background by using values from the no-bacteria control. The MIC can be seen as a range in between the concentration where cells do not grow and the concentration where some growth is observed (*see* **Note 2**).

3.1.2 *In Vitro Conjugation Tests Between* Salmonella *Donors and Enterobacteriaceae Recipients to Characterize Plasmids of Interest* (See **Note 3**)

1. Inoculate LB overnight cultures (with the appropriate antibiotics) of each of the donor and recipient strains of interest.
2. Subculture all strains 1:20 in LB without antibiotics and grow for 4 h at 37 °C on a rotating wheel to ensure that strains are at late exponential phase and that they have reached approximately the same density.
3. Centrifuge 1 mL of subcultures separately at 18,000 × *g* for 1 min, discard supernatant, and resuspend in 1 mL of PBS.
4. Dilute cells 100-fold by transferring 10 μL of the cell suspension to 990 μL of PBS.
5. Inoculate 5 mL of LB with 10 μL of donor and 10 μL of recipient dilutions. Grow the mixture overnight at 37 °C on a rotating wheel.
6. Enumerate population sizes of mated cultures by plating dilutions of the cultures on LB or MacConkey plates with one of three antibiotic mixtures (plasmid-specific antibiotic to select for donors and transconjugants; recipient-specific antibiotic to select for recipients and transconjugants; both antibiotics to select for transconjugants). To determine how efficiently a plasmid has spread, one can report the fraction of transconjugants divided by recipients including transconjugants. For subsequent analysis of persister-mediated plasmid spread, plasmids that have efficient plasmid spread in vitro will likely also spread in vivo [8].

3.1.3 *Identifying a Suitable Donor Strain in an Animal Model for Typhoidal Salmonellosis*

1. It is first necessary to establish if the donor strain of interest is capable of colonizing systemic sites of the animal model following intraperitoneal or intravenous infection (*see* **Note 4**) (and the gut murine gut lumen; *see* Subheading 3.1.4). For the donor strain, set up overnight cultures in LB containing the appropriate antibiotic (e.g., 50 μg/mL of streptomycin for *S.* Tm SL1344). Incubate at 37 °C on a rotating wheel for 12 h.
2. Transfer 1 mL of each culture into separate Eppendorf tubes, spin down the bacteria (18,000 × *g* for 2 min at 4 °C), remove the supernatant and resuspend in 1 mL of ice-cold PBS. Centrifuge at 18,000 × *g* for 2 min at 4 °C and remove the supernatant. Repeat two more times for a total of three washes. Resuspend in 1 mL of PBS.
3. Dilute washed cultures to 1:100,000 to generate the inoculum containing approximately 10^3 CFU in 100 μL of PBS. Ensure that there is enough volume for each mouse (100 μL inoculum with excess). Plate 50 μL of inoculum, diluted to yield approximately 100–1000 CFU, to selective media (e.g., MacConkey containing 50 μg/mL of streptomycin) to estimate the total population size.

4. Administer 100 μL of inoculum to mice by intraperitoneal injection using a sterilized 26G 0.45 × 12 mm needle.
5. At days 2, 3, and 4 postinfection, administer 1.5 mg ceftriaxone dissolved in 100 μL of PBS by intraperitoneal injection and collect feces to determine if reseeding of systemic *S.*Tm into the gut lumen occurs before the cessation of antibiotic therapy. Weigh the total volume of the tube before and after feces collection to determine feces weight. Homogenize feces for 1 min at 25 Hz using a TissueLyser. Plate the feces to selective MacConkey media.
6. At day 5 postinfection, euthanize by cervical dislocation following anesthesia. Aseptically remove the organs of interest (e.g., liver and spleen), transfer to Eppendorf tubes containing PBS and a 5 mm steel ball, and homogenize for 2.5 min at 25 Hz using a TissueLyser. Plate 50 μL of the homogenate to selective media. Incubate at 37 °C overnight and count CFU to confirm successful colonization of systemic sites by the donor strain.

3.1.4 Identifying a Suitable Recipient Strain in an Animal Model

1. Similarly to Subheading 3.1.3, it is also necessary to verify that the recipient strain of interest is capable of colonizing the murine gut lumen following oral infection. For the recipient strain, set up overnight cultures in LB with the appropriate antibiotic, and incubate overnight at 37 °C on a rotating wheel.
2. On the same day, pretreat mice using an oral gavage feeding needle with an antibiotic to which the recipient is resistant (e.g., 25 mg streptomycin; 20 mg ampicillin; or 10 mg kanamycin) (*see* **Note 5**). Treat the mice with antibiotics 24 h before the planned infection time of the recipient strain. Leave one group of mice unpretreated to determine if the recipient strain can colonize an unperturbed gut.
3. Subculture cells 1:20 in 2 mL of LB. Incubate for 4 h at 37 °C on a rotating wheel.
4. Centrifuge 1 mL of the subculture at 18,000 × *g* for 2 min at 4 °C. Remove the supernatant and resuspend in 1 mL of PBS. Aliquot 50 μL of inoculum for each mouse.
5. Infect mice using an oral gavage feeding needle.
6. Monitor populations in the gut lumen by collecting feces and plating to selective media containing appropriate antibiotics. Count CFU to confirm successful colonization of the gut lumen by the recipient strain. Successful colonization for at least 7 days is recommended for assessing plasmid transfer dynamics associated with reseeding.

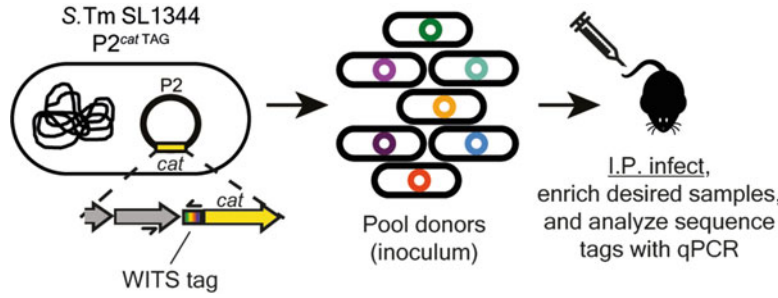

Fig. 1 WITS tag locus on the P2 conjugative plasmid and preparation of the mixed inoculum. Tags coupled to a chloramphenicol marker (*cat*) are introduced into the plasmid of choice (here P2 of SL1344) using the lambda red system. Primers specific to the unique tag (Table 1) and the flanking gene in the WITS locus (*ydgA*) are indicated as one-sided arrows. Donors bearing a unique tag are mixed at equal ratios to generate an inoculum, which is then intraperitoneally (I.P.) injected into mice

3.1.5 Tagging of Plasmids with WITS Tags Coupled to Antibiotic Resistance Markers

1. Find a fitness-neutral location on the plasmid (e.g., regions flanking accessory plasmid genes, we used colicin 1b on plasmid pCol1b9 (also called P2) of SL1344; NCBI accession number NC_017718.1).
2. Design primers that amplify regions upstream and downstream of the WITS constructs coupled to either kanamycin or chloramphenicol resistance cassettes (Fig. 1) [7, 19, 20]:
   (a) FW primer: [>40 nucleotides upstream of insertion locus in conjugative plasmid] + [upstream region of the WITS locus].
   (b) RV primer: [reverse complement of >40 nucleotides downstream of insertion locus in conjugative plasmid] + [downstream region of the WITS locus].

   This PCR can be performed using DNA from any WITS-tagged strain coupled to the chloramphenicol resistance cassette as a template (e.g., strains from [7, 19, 20]).

   For example, for tagging the pCol1b9 plasmid with chloramphenicol WITS, the following primers were used [7]:
   (a) FW primer: [GCA TGA TAA TAA TAA TCA ATA ACA ATA AGC TGT GTC ACG TTT ACA TCA T] + [GGC TGT CCG CAA TGG GT].
   (b) RV primer: [AAG GGT AAT GGC GGA AGC CGG ATA CCC AGC CGC CAG AGA A] + [AT CGA ACA TAT CCC TTC CTT A].
3. Perform lambda red-mediated recombineering according to previously published protocols [26] to insert the WITS construct into the desired locus. Validate insertion with genotyping PCR.

4. If the recombineered strain is *S.*Tm, use P22 transduction to transfer the resistance cassette into a clean *S.*Tm strain (*see* **Note 6**).
5. If mechanistic information is desired about bacterial genes involved in persister formation, the lambda red system can also be used to inactivate candidate genes [26].

3.2 Infection of Mice with the Donor and Antibiotic Treatment Schedule to Yield Tissue Lodged Persisters

3.2.1 Generation of Mixed WITS Inoculum

1. The mixed WITS inoculum preparation will change depending on which process should be captured. WITS-tagged strains should be mixed equally (i.e., without any addition of untagged isogenic strains), if one expects very rare events (*see* **Note 7**). Here, we describe the protocol for such a rare reseeding/transfer event using donors carrying WITS-tagged plasmids (Fig. 1).
2. Set up overnight cultures of each S.Tm WITS donor (i.e., WT S.Tm SL1344 carrying WITS-tagged P2 plasmids [7] in LB containing 15 μg/mL of chloramphenicol or 50 μg/mL of kanamycin, corresponding to the antibiotic resistance cassette that is linked to the WITS tag on the plasmid). If needed (i.e., in cases of frequent reseeding or transfer events), separately set up overnight cultures of *S.*Tm carrying untagged P2 in LB containing 50 μg/mL of streptomycin. Incubate cultures at 37 °C on a rotating wheel for 12 h (*see* **Note 8**).
3. Transfer 1 mL of each culture into separate Eppendorf tubes, spin down the bacteria (18,000 × *g* for 2 min at 4 °C), remove the supernatant and resuspend in 1 mL of ice-cold PBS. Centrifuge at 18,000 × *g* for 2 min at 4 °C and remove the supernatant. Repeat two more times for a total of three washes. Finally, resuspend in 1 mL of PBS.
4. Combine 100 μL of each *S.*Tm WITS strain into one Eppendorf tube and vortex well. If a diluted WITS inoculum is desired (expecting a more subtle bottleneck or less rare event), combine this mixed culture with *S.*Tm untagged culture, to achieve the desired dilution of *S.*Tm WITS to *S.*Tm untagged.
5. Dilute the washed culture 1:100,000 to generate the inoculum containing approximately 10^3 CFU in 100 μL PBS.
6. To confirm the correct inoculum size, plate 50 μL of inoculum, diluted to yield approximately 100–1000 CFU, to (1) one MacConkey plate containing 50 μg/mL chloramphenicol or 50 μg/mL kanamycin to estimate the *S.*Tm WITS population size, and to (2) 50 μg/mL streptomycin to estimate the total population size, if an untagged strain is used. Incubate overnight at 37 °C and count colonies to confirm the correct inoculum size and to confirm the desired ratio of *S.*Tm WITS mix to *S.*Tm untagged bacteria.

7. To experimentally determine the WITS distribution in the input pool, take 100 µL of inoculum (undiluted) and mix with 2 mL of LB containing 15 µg/mL chloramphenicol or 50 µg/mL kanamycin, corresponding to the antibiotic specific for the WITS-tagged plasmids. Incubate at 37 °C overnight (*see* **Note 9**). Centrifuge the overnight cultures at 18,000 × *g* for 2 min, remove supernatant, and store pelleted material at −20 °C (*see* **Note 10**).

3.2.2 Animal Infection and Antibiotic Treatment Schedule

1. Administer 100 µL of the inoculum prepared in Subheading 3.2.1 by intraperitoneal injection using a sterilized 26G 0.45 × 12 mm needle (Fig. 1). Mice can also be infected intravenously by injecting the same volume of inoculum into the tail vein (*see* **Note 11**).
2. At day 2 postinfection, administer 1.5 mg of ceftriaxone dissolved in 100 µL PBS (*see* **Note 12**) by intraperitoneal injection. Collect feces and plate as in Subheading 3.1.3. Transfer mice to new cages to minimize the impact of coprophagy (*see* **Note 13**). Repeat administration of ceftriaxone and feces collection on day 3 and day 4 postinfection.
3. In control animals, analyze the persister reservoir size (as described in Subheading 3.2.3) and WITS distribution (*see* Subheading 3.4) (*see* **Note 14**) after the cessation of antibiotic treatment (e.g., day 5 postinfection).

3.2.3 Euthanasia of Mice and Analysis of Persister Reservoirs and Population Sizes

1. At required endpoint, collect feces as described above. Anesthetize mice before euthanizing the animals by cervical dislocation or CO_2 asphyxiation.
2. *S.*Tm persisters have been isolated from many different organs [7]. The primary reservoirs of interest for the intraperitoneal or intravenous infection model are the liver, spleen, and gall bladder. After euthanization, aseptically remove the organs of interest and transfer to Eppendorf tubes containing PBS and a 5 mm steel ball.
3. Homogenize organs and feces for 2.5 min at 25 Hz using a TissueLyser. Split homogenates into two aliquots, one for determining the total densities of plateable bacteria and one for determining the WITS distribution (Subheading 3.4) after enrichment (Subheading 3.2.4) (*see* **Note 10**).
4. Dilute appropriately and plate 50 µL of the homogenate to MacConkey plates containing 15 µg/mL or chloramphenicol or 50 µg/mL or kanamycin to select for donors, and if necessary 50 µg/mL of streptomycin to enumerate the total *S.*Tm population (if untagged strains are used). Incubate at 37 °C overnight.

5. Count CFU and express data as CFU of donors per organ to report differences between treatment groups (e.g., compare a ceftriaxone-treated group to a PBS-treated control), bacterial strains (e.g., for S.Tm an SPI-1- and SPI-2-deficient avirulent mutant can be compared to a wild type), or transgenic lines (e.g., to investigate the role of host factors).

3.2.4 Enrichment of S. Tm WITS from Fecal or Organ Homogenates

1. Inoculate enrichment cultures by combining 100 μL homogenates (from Subheading 3.2.3) with 2 mL of LB containing 15 μg/mL chloramphenicol or 50 μg/mL kanamycin corresponding to the antibiotic resistance gene used to tag the plasmid, and incubate at 37 °C overnight (*see* **Note 9**).
2. Centrifuge enrichment cultures at 18,000 × *g* for 2 min at 4 °C. Remove supernatant.
3. Extract chromosomal DNA from bacterial pellets (derived from starting inoculum, fecal populations, or organ homogenates) using any standard DNA extraction procedure.
4. Store DNA at −20 °C for future analysis, or use immediately for qPCR (Subheading 3.4).

3.3 Introduce Recipient Strains by Oral Gavage and Analysis of Transconjugant Formation in the Gut Lumen

3.3.1 Infecting Mice Harboring Tissue-Lodged Persisters with Recipient Strains

1. Prepare overnight cultures of the desired recipient strain in LB (here, we describe the use of *S.*Tm 14,028 bearing a kanamycin resistance cassette in the chromosome since the donor contains a chloramphenicol resistance cassette on the plasmid) with the appropriate antibiotics at 37 °C on a rotating wheel. Overnight cultures should be set up the day before infection of the recipients, corresponding to day 6 postintraperitoneal or intravenous infection of the donors (*see* **Note 15**).
2. Subculture the recipient strain 1:20 in 2 mL of LB. Incubate for 4 h at 37 °C on a rotating wheel.
3. Centrifuge 1 mL of cells at 18,000 × *g* for 2 min at 4 °C, remove the supernatant and resuspend in 1 mL of PBS. Aliquot 50 μL of inoculum for each mouse.
4. Take mice from step in Subheading 3.2.2 and infect them orally with the recipient strain using an oral gavage feeding needle.
5. After infection of the recipient, cage each mouse separately.
6. Monitor the populations in the gut lumen daily by collecting feces. Each sample should be plated on MacConkey plates with the appropriate antibiotics to select for: plasmid-specific antibiotic to select for donors and transconjugants; recipient-specific antibiotic to select for recipients and transconjugant; both antibiotics to select for transconjugants. Transfer efficiency can be reported as a proportions of transconjugants, calculated by dividing the transconjugant population by the sum of transconjugants and recipients.

7. When appropriate (*see* **Note 16**), take enrichments of donors (plasmid-specific enrichment) and/or transconjugants (plasmid-specific enrichment plus the antibiotic for the recipients).

3.3.2 Euthanasia of Mice at the End of the Experiment, Analysis of Conjugation Dynamics, and the Formation of Reservoirs of S. Tm

1. At the desired time point, euthanize mice. Anesthetize mice before euthanizing the animals by cervical dislocation or CO_2 asphyxiation.
2. Perform harvesting of organs as in Subheading 3.2.3, but in this case, enumerate homogenates on three different antibiotic combinations: plasmid-specific antibiotic to select for donors and transconjugants; recipient-specific antibiotic to select for recipients and transconjugants; both antibiotics to select for transconjugants. The proportion of transconjugants can be reported in the feces as well as in each organ.
3. Perform enrichment and DNA extraction of homogenates as in Subheading 3.2.4, but in this case perform two types of enrichments. Enrich for donors using the plasmid-specific antibiotic (WITS-coupled resistance cassette) and transconjugants using both the plasmid-specific antibiotic resistance and the recipient-specific resistance cassette.

3.4 Quantification of WITS Tag Abundance by qPCR

3.4.1 qPCR Analysis of WITS Tag Abundance

1. For each sample, prepare the number of qPCR reactions corresponding to the number of WITS tags used. We used seven different WITS tags, with each reaction using one of the seven WITS forward primers and the common *ydgA* reverse primer (Table 1). Prepare all reactions in duplicate.
2. In parallel, prepare reactions using 5× tenfold dilutions starting at 50 ng/μL of DNA extracted from a culture of a WITS-bearing strain (from Subheading 3.2.4). Data from these reactions are used to generate a standard curve to allow the quality of the qPCR run to be determined, and to define the detection limit (*see* **Note 17**).
3. Perform qPCR according to the amplification protocol below (*see* **Note 18**):

94 °C	10 min	
94 °C	15 s	40×
61 °C	30 s	
72 °C	20 s	

We suggest to perform the qPCR reaction in a 96-well plate format where each reaction consists of a 20 μL volume containing 10 μL of Roche FastStart Universal SYBR Green

Master (Rox), 5 μL of 10 mM primer (Table 1), and 5 μL of genomic DNA (approximately 50 ng/μL) of the desired sample.

4. For each duplicate CT value derived from qPCR, take the mean of the two values, then fit to the standard curve generated from the standard reactions. Take the antilogarithm of this value to determine the copy number.
5. Express the relative abundance of each WITS tag from a single sample as a proportion by dividing the copy number of one tag by the sum of all copy numbers of all tags from that sample.

3.4.2 Analysis of the Population Structure and Detection of Bottlenecks or Rare Events

1. For each mouse, analyze the relative proportions of each barcoded strain from Subheading 3.4.1 from each organ. Within each sample, rank the tags from most to least abundant, not taking into consideration which tag is which.
2. In each organ (separately for each type of enrichment), treat all the most abundant tags as individual values from one group. Repeat this for the second most abundant tag, and for each tag grouping. Plot these values on a graph. The skew of tags can be quantified using statistics (paired nonparametric testing with multiple comparison corrections recommended). Another informative measure is the number of tags that are present above the detection limit. The detection limit can be assigned due to the limit of linearity of qPCR (which is observed below a certain concentration of target DNA). This can be estimated by taking the most diluted sample of the DNA standard in each qPCR run and observing the CT value where this occurs.

3.5 Modifications to the Protocol for Alternate Applications: Oral Infection with S. *Tm Donor Strains as an Example*

Thus far, we have described an approach to analyze persister reservoirs and dynamics in a typhoidal experimental model. This approach allows the gut luminal microbiota to remain more intact, and ensures that no persisters can be formed in the gut lumen. However, a disadvantage is that it does not capture processes associated with the normal fecal–oral route of colonization of *Salmonella* spp. To address this, the experimental model above can be modified in key steps. Here, we present an example of how to modify the above protocol to accommodate an oral infection model using SL1344 P2$^{cat\ TAG}$ strains. If deviations to the protocol are not described here, proceed as mentioned above. Please note that this is an example to serve as a basis as how to modify the above protocol to address slightly different experimental questions in a variety of relevant contexts.

1. The route of infection will be different (modify Subheadings 3.2.1 and 3.2.2):
   (a) Pretreat mice with 25 mg streptomycin using an oral gavage feeding needle to open a niche for *S.*Tm colonization 24 h before the desired time of infection (*see* **Note 19**).

(b) Inoculate *S*.Tm SL1344 WITS donors as in Subheading 3.2.1, and the following day, subculture 1:20 in 2 mL of LB without antibiotics for 4 h.

(c) Transfer 1 mL of culture into separate Eppendorf tubes. Centrifuge bacteria (18,000 × *g* for 2 min at 4 °C), remove the supernatant and resuspend in 1 mL of ice-cold PBS.

(d) Pool isogenic WITS strains together (either diluted within untagged wild type or as equal ratios) and aliquot 50 μL for infection.

(e) Infect mice using an oral gavage feeding needle (5×10^7 CFU doses). Enumerate the inoculum by selective plating and enrich the inoculum for qPCR analysis.

2. The treatment regime will be different (modify Subheading 3.2.2):
   (a) To kill off susceptible (growing) *S*.Tm cells and thereby enrich for persister populations, administer 3 mg of ciprofloxacin as a 100 μL volume (*see* **Note 20**) using a sterilized oral gavage needle on day 2 postinfection. In order to ensure the clearance of the entire gut luminal *S*.Tm population, ciprofloxacin should be administered daily for at least three subsequent days. Enumerate fecal populations by selective plating to verify the successful elimination of susceptible donors from the gut lumen. To prevent relapse of persisters into the gut lumen, 2 g/L of ampicillin can be added to the drinking water from day 3 on, if the *S*.Tm population is ampicillin sensitive (*see* MIC testing in Subheading 3.1.1). Mice should be transferred into fresh cages on day 2, 3, and 4 (after each dose of ciprofloxacin) to prevent reinfection from coprophagy (*see* **Note 13**).
3. The persister reservoirs will be in different primary locations. In the oral model, the primary locations of tissue reservoirs are in the mesenteric lymph node and cecal tissue (compared to spleen, liver, and gall bladder for the intravenous infection model). Therefore, in addition to organs that can be aseptically removed as in Subheading 3.2.3, the cecal tissue should be collected. In order to minimize contamination from luminal microbes, a gentamycin protection assay is required.
   (a) Open the cecal tissue longitudinally (*see* **Note 21**) and wash the tissue three times in a petri dish with PBS, changing dishes and drying the tissue in between each wash.
   (b) Transfer the cecal tissue into 1 mL of PBS containing 400 μg/mL of gentamycin and set a timer for 30 min (*see* **Note 22**).

(c) After 30 min, transfer the tissue into a well of a 24-well plate filled with 2 mL of PBS. Shake the tissue with forceps for 15 s and incubate for 30 s. After the 45 s have passed, move the tissue into the next well and repeat until six to nine washes are completed. Transfer the tissue into an Eppendorf tube containing PBS and a 5 mm steel ball.

(d) Process the cecal tissue as the other organs (Subheading 3.2.3).

4 Notes

1. These tests are important for two reasons: (1) to ensure that the strains used are not resistant to antibiotics that will be used to enrich for persisters (this can be particularly relevant for clinical isolates); and (2) to ensure there is no cross-reactivity of some antibiotic resistances, or no unexpected antibiotic resistance. Given these criteria, all antibiotics used in these MIC tests should reflect all antibiotics used experimentally for treatment or selection.
2. Selecting a donor strain with the appropriate resistance is an important aspect of experimental design. Here, we describe the use of a streptomycin-resistant *S.*Tm strain which is suitable for use in the streptomycin pretreatment mouse model. If an alternate design is needed, pretreatment with either kanamycin (10 mg p.o.) or ampicillin (20 mg p.o) is also suitable.
3. Experiments with ESBL plasmids suggest that in vitro conjugation assays are handy to identify donor–recipient pairs with the desired conjugation frequency in vivo [8]. To investigate plasmid transfer dynamics in vivo, one should first ensure that plasmid transfer proceeds in vitro. Find a naturally occurring resistance marker on the plasmid of interest, or insert a selectable marker in a fitness neutral location using the lambda-red method [26] to select for the plasmid [3, 6, 7]. Introduce a second resistance marker by lambda-red insertion into the chromosome of the desired recipient strain, or by transforming a small nonconjugative resistance plasmid. In order to study in vivo *Salmonella* persisters, at least one of the two strains should be *Salmonella*, and the other should be a *Salmonella* strain or other Enterobacteriaceae. The example we present in this chapter describes a *S.*Tm donor with a *S.*Tm recipient. However, equivalent protocols apply if *E. coli* or other Enterobacteriaceae are chosen as either donor or recipient.
4. The choice of animal model is an important aspect of experimental design. C57BL/6 mice are inherently susceptible to *S.* Tm infection and are suitable for short term experiments (up to

4 days postinfection), while 129S6/SvEvTac are resistant to *S.* Tm infection as they bear a functional allele of Slc11a1, and are thus more suited to long term experiments. Here, we used 129S6/SvEvTac to investigate persister formation over longer periods of time. However, it is possible to perform these experiments in susceptible mouse lines (e.g., C57BL/6) since a low systemic inoculum size is used paired with antibiotic treatment, which is used to eliminate vegetative bacteria and select for persisters. Since the antibiotic treatment is started at day 2 postinfection, the antibiotic will prevent deadly courses of the systemic infection.

5. This antibiotic pretreatment often enhances gut luminal growth of the recipient strains. This is equivalent to the well-established streptomycin mouse infection model for *S.*Tm SL1344 infections [27]. In some cases, we have observed that efficient recipient growth occurs even without a previous antibiotic pretreatment.
6. P22 transduction of genes that have been replaced by resistance cassettes is recommended, to remove the influence of unintended changes introduced elsewhere in the genome, and to ensure pKD46 is cleared from the recipient strain. Conjugation of modified plasmids can be performed if transduction is not possible; however, then a selectable marker in the target strain is required to ensure conjugation.
7. This protocol describes the use of undiluted WITS strains to look at plasmid transfer after antibiotic treatment relapse, as this is a relatively rare event. However, the dilution of WITS relative to an isogenic parent strain should be used if expecting a more common event. If all tags are always present at the same abundance, or if no tags are found to be present, consider diluting the WITS strains with an untagged isogenic strain.
8. Incubation of overnight cultures for precisely 12 h in a programmable incubator minimises variations in cultures that may arise from shorter or longer incubation times, which may influence the dynamics of infection in vivo.
9. Enrichment cultures are easily prepared in a 2 mL volume in Eppendorf tubes, as these can be immediately centrifuged following overnight incubation at 37 °C. However, inoculating 5 mL of cultures for incubation on a rotating wheel at 37 °C overnight can yield a higher degree of enrichment, allowing for greater sensitivity in downstream qPCR.
10. Enrichment cultures of infection inocula are necessary to establish that each WITS-tagged strain is present at approximately equal amounts in the starting inocula, and thus any differences that arise in the relative amount detected in organ samples is due to dynamics of infection.

11. For infection, prepare sufficient inoculum to allow for wasted volume when drawing inoculum into the needle and removing air bubbles prior to injection.
12. Ceftriaxone for intraperitoneal injection is dissolved in PBS. We recommend to prepare ceftriaxone to a concentration of 15 mg/mL and filter sterilize immediately before treatment.
13. Mice often engage in coprophagy, which can lead to transmission of bacterial populations from the gut lumen of one mouse to another. This is particularly important when analysing reseeding of the gut lumen, which is a stochastic process. To minimize this, fresh cages can be used each day, or mice can be placed on steel grid floors during this phase of the experiment to prevent access to the faecal pellets. To prevent coprophagy completely, consider housing mice individually for the duration of the persister experiment.
14. To check for bottlenecks in reservoir formation and/or survival during antibiotic therapy, qPCR of WITS tags can be performed with varying dilutions of WITS within untagged wild type from organ homogenates taken from day 2 (before antibiotic treatment) or day 5 (after antibiotic treatment).
15. To prevent reseeding of the gut lumen by donors in host tissues prior to the addition of the recipient strain, ampicillin can be used in the drinking water to suppress donor growth (assuming the use of an ampicillin-sensitive donor strain). In this case, an ampicillin resistant recipient derivative should be used and ampicillin-drinking water can be removed on the day the recipient is introduced.
16. Enrichments are taken at critical time points during the experiment. These time points will depend on experimental design. Ideally, enrichments can be taken each day to resolve subtle dynamics of reseeding and plasmid transfer, but sufficient information can be gathered from WITS analysis at the endpoint (e.g., the day of euthanasia). Comparing the distribution of tags in the transconjugant population and the donor population in the faeces compared to the tag distribution in the organs at the endpoint can inform about reservoir formation and rarity of plasmid transfer dynamics. Additional enrichments from the faeces over time can provide information about multiple relapse events and competing extinction events.
17. For generation of standard curves in qPCR, DNA from any WITS-bearing bacteria can be used as template DNA, along with the corresponding WITS-specific forward primer and common *ydgA* reverse primer.
18. This amplification protocol is optimised for the melting temperature of the qPCR primers shown in Table 1. This will need to be further optimized if different tags or primers are used.

19. Here we use streptomycin pretreatment since SL1344 is streptomycin resistant. Alternate antibiotics can be used if the donor strain is not resistant to streptomycin (e.g., 10 mg of kanamycin; 20 mg of ampicillin).
20. Ciprofloxacin hydrochloride can be used to allow solubility without needing to manually reduce the pH. To avoid precipitation, however, ciprofloxacin should be dissolved (30 mg/mL) and filter-sterilized shortly prior to administration.
21. The cecum can be divided for several purposes. For example, parts can be fixed in paraformaldehyde for later immunofluorescence staining, stored in Optimal Cutting Temperature compound medium for hematoxylin and eosin staining, or frozen in liquid nitrogen for transcriptomic or proteomic analysis. Therefore, approximately a quarter of the cecum should suffice for enumeration by the gentamycin protection assay.
22. Due to the constraints with the 30 min incubation of gentamycin at room temperature (to avoid progressive tissue collapse after removal from the intestine), a limited number of mice is possible per participant. The more people that participate, the more mice can be analyzed.

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References

1. Tenaillon O, Skurnik D, Picard B et al (2010) The population genetics of commensal *Escherichia coli*. Nat Rev Microbiol 8:207–217
2. Diard M, Bakkeren E, Cornuault JK et al (2017) Inflammation boosts bacteriophage transfer between *Salmonella* spp. Science 355:1211–1215
3. Stecher B, Denzler R, Maier L et al (2012) Gut inflammation can boost horizontal gene transfer between pathogenic and commensal Enterobacteriaceae. Proc Natl Acad Sci U S A 109:1269–1274
4. Stecher B, Maier L, Hardt WD (2013) 'Blooming' in the gut: how dysbiosis might contribute to pathogen evolution. Nat Rev Microbiol 11:277–284
5. Bakkeren E, Diard M, Hardt WD (2020) Evolutionary causes and consequences of bacterial antibiotic persistence. Nat Rev Microbiol 18:479–490
6. Wotzka SY, Kreuzer M, Maier L et al (2019) *Escherichia coli* limits *Salmonella* Typhimurium infections after diet shifts and fat-mediated microbiota perturbation in mice. Nat Microbiol 4:2164–2174
7. Bakkeren E, Huisman JS, Fattinger SA et al (2019) *Salmonella* persisters promote the

spread of antibiotic resistance plasmids in the gut. Nature 573:276–280

8. Benz F, Huisman JS, Bakkeren E et al (2020) Clinical extended-spectrum beta-lactamase antibiotic resistance plasmids have diverse transfer rates and can spread in the absence of antibiotic selection. BioRxiv https://www.biorxiv.org/content/10.1101/796243v2
9. Stecher B, Robbiani R, Walker AW et al (2007) *Salmonella enterica* serovar Typhimurium exploits inflammation to compete with the intestinal microbiota. PLoS Biol 5:2177–2189
10. Raffatellu M, George MD, Akiyama Y et al (2009) Lipocalin-2 resistance confers an advantage to *Salmonella enterica* serotype Typhimurium for growth and survival in the inflamed intestine. Cell Host Microbe 5:476–486
11. Winter SE, Thiennimitr P, Winter MG et al (2010) Gut inflammation provides a respiratory electron acceptor for *Salmonella*. Nature 467:426–429
12. Lawley TD, Bouley DM, Hoy YE et al (2008) Host transmission of *Salmonella enterica* serovar Typhimurium is controlled by virulence factors and indigenous intestinal microbiota. Infect Immun 76:403–416
13. Helaine S, Cheverton AM, Watson KG et al (2014) Internalization of *Salmonella* by macrophages induces formation of nonreplicating persisters. Science 343:204–208
14. Diard M, Sellin ME, Dolowschiak T et al (2014) Antibiotic treatment selects for cooperative virulence of *Salmonella* Typhimurium. Curr Biol 24:2000–2005
15. Claudi B, Sprote P, Chirkova A et al (2014) Phenotypic variation of *Salmonella* in host tissues delays eradication by antimicrobial chemotherapy. Cell 158:722–733
16. Kaiser P, Regoes RR, Dolowschiak T et al (2014) Cecum lymph node dendritic cells harbor slow-growing bacteria phenotypically tolerant to antibiotic treatment. PLoS Biol 12: e1001793
17. Vulin C, Leimer N, Huemer M et al (2018) Prolonged bacterial lag time results in small colony variants that represent a sub-population of persisters. Nat Commun 9:4074
18. Liu Y, Tan S, Huang L et al (2016) Immune activation of the host cell induces drug tolerance in *Mycobacterium tuberculosis* both *in vitro* and *in vivo*. J Exp Med 213:809–825
19. Grant AJ, Restif O, McKinley TJ et al (2008) Modelling within-host spatiotemporal dynamics of invasive bacterial disease. PLoS Biol 6: e74
20. Kaiser P, Slack E, Grant AJ et al (2013) Lymph node colonization dynamics after oral *Salmonella* Typhimurium infection in mice. PLoS Pathog 9:e1003532
21. Lam LH, Monack DM (2014) Intraspecies competition for niches in the distal gut dictate transmission during persistent *Salmonella* infection. PLoS Pathog 10:e1004527
22. Maier L, Diard M, Sellin ME et al (2014) Granulocytes impose a tight bottleneck upon the gut luminal pathogen population during *Salmonella* Typhimurium colitis. PLoS Pathog 10:e1004557
23. Hausmann A, Bock D, Geiser P et al (2020) Intestinal epithelial NAIP/NLRC4 restricts systemic dissemination of the adapted pathogen *Salmonella* Typhimurium due to site-specific bacterial PAMP expression. Mucosal Immunol 13:530–544
24. Nguyen BD, Cuenca VM, Hartl J et al (2020) Import of aspartate and malate by DcuABC drives H2/fumarate respiration to promote initial *Salmonella* gut-lumen colonization in mice. Cell Host Microbe 27:922–936.e6
25. Gonzalez-Escobedo G, Marshall JM, Gunn JS (2011) Chronic and acute infection of the gall bladder by *Salmonella* Typhi: understanding the carrier state. Nat Rev Microbiol 9:9–14
26. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 97:6640–6645
27. Barthel M, Hapfelmeier S, Quintanilla-Martinez L et al (2003) Pretreatment of mice with streptomycin provides a *Salmonella enterica* serovar Typhimurium colitis model that allows analysis of both pathogen and host. Infect Immun 71:2839–2858

Chapter 18

Studying Antibiotic Persistence During Infection

Charlotte Michaux, Séverin Ronneau, and Sophie Helaine

Abstract

This chapter contains the latest version of essential protocols established to study *Salmonella* persisters during macrophage infection. These methods, which can be applied to other pathogens, allow researchers to quantify, visualize, and characterize bacterial persisters within a population and within immune cells consistent with the recent consensus statement published by the research community working on antibiotic persistence (Balaban et al, Nat Rev Microbiol 17:441–448, 2019). These protocols notably allow the discrimination between tolerance and persistence during infection, which is essential to clarify which phenomenon is actually reported. Methods described in this chapter may contribute to the determination of key bacterial and host genes that contribute to antibiotic persistence.

Key words Pathogen, Persisters, Infection, Macrophages, Tolerance, Persistence, Single-cell

1 Introduction

Numerous bacterial pathogens colonize host tissues and establish long-lasting infections by avoiding host immune defenses and antibiotic treatment. Several studies have suggested that antibiotic persisters are important in this process. Antibiotic persisters represent a subpopulation of bacteria, which, through growth arrest, survive exposure to concentrations of antibiotics otherwise lethal for the rest of the population (for review [2]). Contradictory results produced by various laboratories have led to confusions and controversies within the field, most likely due to a failure to distinguish between antibiotic persistence and antibiotic tolerance. Antibiotic tolerance describes the ability of a whole bacterial population to survive longer antibiotic treatments. This is often due to particular environmental conditions or genetic mutations that impair bacterial growth [1]. In trying to dissect antibiotic persistence, scientists use tools, conditions or mutations that often alter the growth rate

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of the population and thereby compromise their observations. Bacterial persistence is, by definition, a transient phenotype arising only in a subpopulation, which makes it extremely challenging to study. The development of novel methods, notably single-cell approaches, has gradually facilitated the study of antibiotic persisters. It is essential to document validated and systematic protocols that allow characterization of antibiotic persisters, without the confounding factor of general tolerance.

Over the last decade, our laboratory has pioneered methods to track, quantify, and characterize antibiotic persisters during macrophage infection by *Salmonella* [3]. These tools and protocols have helped us to discover that persisters (i.e., bacteria that survive antibiotic exposure during macrophage infection and regrow after release from host cells in the absence of drugs) emerge from a non-growing population. These non-growing bacteria remain transcriptionally and translationally active during infection, allowing them to reprogram macrophage immune responses [4].

These methods are relevant to study persister biology and have been updated since the previous edition of this book [5]. In the current chapter, we first describe our protocols to quantify the frequency of antibiotic persisters within a bacterial population in vitro (in complex laboratory medium) and in vivo (during macrophage infection). We then describe methods to visualize, identify, and isolate growing and non-growing bacteria using fluorescence dilution. Altogether, these protocols are key to determine whether the phenomenon of tolerance or persistence is observed, and which is responsible for the survival of bacteria upon antibiotic exposure in vitro and in vivo. Lastly, we detail how a global approach, dual RNAseq, can be used to understand antibiotic persistence during infection from both the host and bacterial perspective.

2 Materials

2.1 Quantification of Antibiotic Persisters In Vitro and In Vivo

1. Sterile and dry Luria–Bertani (LB) agar plates.
2. 200 mL sterile LB (20 mL per strain tested).
3. 50 mL sterile phosphate buffer solution (PBS 1×).
4. Sterile 96-well plates.
5. Multipipette, 8-channels, 20–200 μL.
6. 50 mg/mL cefotaxime stock solution: Dissolve 0.5 g cefotaxime sodium salt in 10 mL of dH_2O. Filter-sterilize the solution using a 0.2 μm syringe filter unit into ten sterile 1.5 mL microfuge tubes. Store aliquots at −20 °C and thaw before use.
7. L-shaped cell spreaders (seven per strain tested).

2.2 Infection of Bone-Marrow Macrophages [5]

1. Aliquots of fetal calf serum (FCS) and mouse serum: Thaw stocks which are kept at −80 °C and aliquot them (50 mL for FCS and 1 mL for mouse serum). Store aliquots at −20 °C.
2. Infection medium: 500 mL of DMEM with high glucose and glutamine, 5 mL of 100 mM sodium pyruvate, 5 mL of 1 M HEPES, 50 mL of heat inactivated FCS, 0.5 mL of 0.05 M β-mercaptoethanol. Filter and store at 4 °C.
3. 0.1% Triton X-100 solution: Dilute 500 μL of 10% filtered Triton X-100 in 50 mL of sterile PBS.
4. Sterile 6-wells plates.

2.3 Visualization of Heterogeneity in a Bacterial Population During Infection

1. Materials listed in Subheading 2.1.
2. Strains to be tested that are transformed with the pFCcGi plasmid [5].
3. 20% L-arabinose stock solution: Dissolve 2 g arabinose in 10 mL of dH_2O. Filter-sterilize the solution using a 0.2 μm syringe filter unit into a sterile plastic tube. Store at room temperature (RT).
4. MgMES minimal medium: 170 mM 2-(N-morpholino)ethanesulfonic acid (MES) at pH 5.0, 5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 1 mM KH_2PO_4, 8 mM $MgCl_2$, 38 mM glycerol, and 0.1% casamino acids. Filter-sterilize the medium.
5. FACS tubes with a cell strainer cap.
6. 3% paraformaldehyde (PFA) solution: Mix 6 g PFA with 200 mL 1× PBS in a beaker and stir on a hotplate at 60 °C in a chemical fume hood for 30 min for complete PFA dissolution. Aliquot the solution in sterile plastic tubes. Store aliquots at −20 °C and thaw before use. Utilization of a safety mask is highly recommended when preparing the PFA solution.
7. 50 mg/mL carbenicillin stock solution: Dissolve 0.5 g carbenicillin disodium salt in 10 mL of dH_2O. Filter-sterilize the solution using a 0.2 μm syringe filter unit into 10 sterile 1.5 mL microfuge tubes. Store aliquots at −20 °C and thaw before use.
8. 100 mg/mL gentamicin stock solution: Dissolve 1 g gentamicin sulphate salt in 10 mL of dH_2O. Filter-sterilize the solution using a 0.2 μm syringe filter unit into ten sterile 1.5 mL microfuge tubes. Store aliquots at −20 °C and thaw before use.

2.4 Bacterial Persistence Using DualRNAseq

1. Materials listed in Subheading 2.2.
2. Materials listed in Subheading 2.3 with the exception of PFA and cefotaxime.
3. Materials required for RNA extraction and cDNA library preparation such as mirVana Kit (Thermofisher), Ribozero + rRNA

depletion kit (Illumina), DNAse I (Thermofisher), SUPERaseIN (Invitrogen), Phase-Lock tubes (5PRIME), Nuclease-free water (NEB), RotiAqua P:C:I 25:24:1 (CarlRoth), AR grade absolute ethanol (Sigma Aldrich), 3 M sodium acetate pH 6.5, Antarctic phosphatase (NEB), T4 polynucleotide kinase (NEB), poly(A) polymerase (NEB), cap-clip acid pyrophosphatase (Cellscript), M-MLV reverse transcriptase (NEB), Agencourt AMPure XP kit (Beckman Coulter Genomics).

4. Specific laboratory equipment required for RNA extraction and cDNA library preparation such as a sonicator bath, a Qubit measurement device (Thermofisher), a nanodrop, access to a Bioanalyzer using high sensitivity DNA chips and the corresponding kit for reagents (Agilent), access to a NextSeq500 platform or any high-throughput RNA sequencing platform.

3 Methods

3.1 Studying Antibiotic Persistence In Vitro (Fig. 1a)

1. Prepare the overnight (ON) cultures. For each strain of interest use a sterile 50 mL plastic tube containing 10 mL of LB inoculated with a single colony (obtained by streaking out the strains from glycerol stocks onto an LB agar plate).
2. Grow at 37 °C for 16 h in a shaking incubator at 200 rpm.
3. In new sterile 50 mL plastic tubes, dilute 1 mL of the ON cultures in 10 mL of fresh LB medium.
4. Incubate at 37 °C for 30 min in a shaking incubator at 200 rpm (*see* **Note 1**).
5. During this time, pipette 180 µL of sterile PBS into each well of a 96-well plate, using a multichannel pipette.

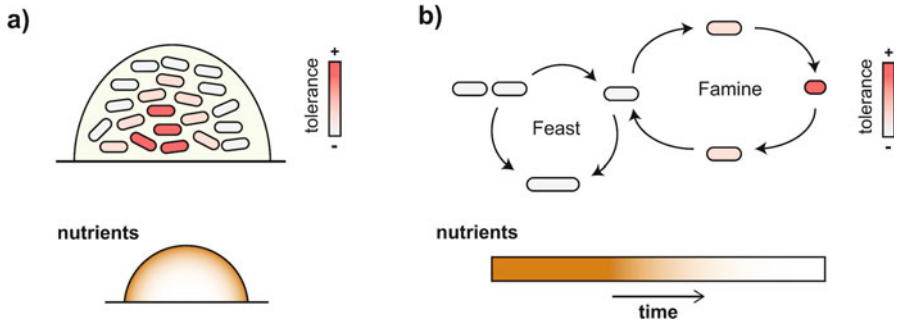

Fig. 1 Quantification of persisters in vitro and in vivo. Following exposure to bactericidal antibiotics, most bacteria are killed rapidly (susceptible cells) whereas a subpopulation is killed at a much slower rate (persister cells). We routinely assess the frequency of persister cells in vitro (in complex laboratory medium; (**a**)) and in vivo (during macrophage infection; (**b**)). *ON* overnight culture, *Ops.* opsonization, *Phago.* phagocytosis. *NG* non-growers

6. After the 30 min incubation, add 20 μL of each culture into a 180 μL of PBS containing well of the 96-well plate. This corresponds to the 10^{-1} dilution of the T0 time point.
7. Immediately after taking 20 μL of each culture, add 2.2 μL of a 50 mg/mL cefotaxime (10 μg/mL final) stock solution to each culture. Incubate the tubes at 37 °C, 200 rpm.
8. Make further tenfold serial dilutions (*see* **Note 2**) in the 96-well plate by diluting 20 μL of the suspensions in 180 μL of PBS using a multichannel pipette and mixing up and down five times. Make sure to change tips in between each dilution to avoid contamination from the lowest to the highest dilution.
9. Spread 100 μL of the chosen dilution(s) on LB agar using a disposable spreader (*see* **Note 2**).
10. Repeat the process of sampling, diluting, and plating at the chosen time points to generate killing curves, for example after 2, 4 and 6 h of incubation (T2, T4 and T6).
11. Leave the plates at RT upside down until all the time points are collected and plated.
12. Incubate all LB agar plates ON at 37 °C in a static incubator.
13. Determine the CFU/mL at each time point by counting the dilution with at least ten colonies. Be sure to account for the dilution factor.
14. Normalize the counts by dividing the number of bacteria for each time point by the number of bacteria obtained at T0 for that strain so the value of T0 for each strain is equal to 1.
15. The normalized values are represented on a line chart with the *y*-axis representing the normalized bacterial counts ($\log_{10}$ scale) as a function of time on the *x*-axis. The first killing slope corresponds to killing of the susceptible bacteria during the first few hours whereas the second slope corresponds to the slower killing rate of persisters (*see* **Note 3**).

3.2 Studying Antibiotic Persistence In Vivo

3.2.1 Infection of Bone-Marrow Macrophages (BMM)

1. Seed fresh BMM onto 6-well plates in infection medium (1×10^6 BMM/well or 1.2×10^6 BMM/well if from frozen cells) (*see* **Note 4**).
2. Incubate macrophages ON at 37 °C in a 5% CO_2 incubator.
3. Grow bacteria in a sterile 50 mL plastic tube containing 10 mL of LB media at 37 °C for 16 h in a shaking incubator at 200 rpm.
4. Warm up some infection medium in a sterile 50 mL plastic tube in a 37 °C water bath. Thaw mouse serum on ice.

5. Opsonize the ON cultures at RT for 20 min by mixing together 170 µL of prewarmed infection medium, 45 µL ON bacterial culture, and 20 µL mouse serum. Mix by vortexing.
6. During this time, prepare the infection medium containing the appropriate antibiotic. Keep this medium at 37 °C until use.
7. After the 20 min of opsonization, add 600 µL of prewarmed infection medium to each opsonization reaction immediately before infection. Mix by vortexing.
8. Infect macrophages with 30 µL of opsonized bacteria per well to reach a multiplicity of infection (MOI) of approximately ten (*see* **Note 5**).
9. Centrifuge the plates at RT for 5 min at 110 × *g*.
10. Incubate at 37 °C in a 5% CO_2 incubator for 30 min to allow phagocytosis to occur.
11. Aspirate the medium in the plates and replace with prewarmed infection medium containing the appropriate antibiotic (T0 of the infection).
12. Incubate the plates at 37 °C in a 5% CO_2 incubator.
13. To collect BMM, aspirate the medium, wash three times with PBS at RT, add 500 µL of 0.1% Triton at RT to lyse the macrophages. Leave for 2 min, pipette up and down and collect in 1.5 mL microfuge tubes. Pellet bacteria at RT for 3 min at 17,000 × *g*. Resuspend the bacteria in 1 mL of PBS.

3.2.2 Quantification of Antibiotic Persisters In Vivo (Fig. 1b)

Proceed with the infection of BMM as described in Subheading 3.2.1 following these modifications:

1. When seeding BMM, prepare a different plate for collection of each time point, with two technical replicates per strain per time point. For example, if a strain is compared to the wild type, two 6-wells plates (T0 and T24) should be prepared with 4 wells each (*see* **Notes 6–8**).
2. During opsonization, prepare infection medium containing 100 µg/mL cefotaxime (**step 6** in Subheading 3.2.1).
3. Replace medium with the infection medium containing cefotaxime 100 µg/mL, except for the T0 plates (**step 11** in Subheading 3.2.1). Incubate the T24 plates at 37 °C in a 5% CO_2 incubator for 24 h. Collect BMM from the T0 plates as described in **step 13** in Subheading 3.2.1. Use 100 µL for serial dilutions in sterile PBS and plate appropriate dilutions on LB plates. Incubate ON at 37 °C in a static incubator.
4. Enumerate the T0 plates counting the appropriate dilution.
5. At T24 collect BMM from the T24 plates as described in **step 13** in Subheading 3.2.1. Pellet bacteria at RT for 3 min at 17,000 × *g* and aspirate 900 µL of the supernatant, leaving the last 100 µL for resuspension of the pellet. Resuspend the pellet carefully.

6. Plate everything on LB plates using an L-shape cell spreader.
7. Incubate ON at 37 °C in a static incubator.
8. After enumerating, normalize the counts by dividing the number of bacteria for each time point by the number of bacteria obtained at T0 for that strain so the value of T0 for each strain is equal to 1. Then, normalize the value for each strain to that of the reference strain (*see* **Note 9**).

3.2.3 Tracking and Quantifying Growing and Non-growing Bacteria (Fig. 2)

Proceed with the infection of BMM as described in Subheading 3.2.1 following these modifications:

1. When seeding BMM, prepare a different plate for collection of each time point, with two technical replicates per strain per time point. For example, if a strain is compared to the wild type, three 6-wells plates (T0, T10, T24) should be prepared with 4 wells each (*see* **Notes 6–8**).
2. Grow bacteria containing the pFCcGi plasmid (Fig. 2a) in 10 mL of MgMES medium supplemented with 5 μL of 100 mg/mL carbenicillin (50 μg/mL final) and 100 μL of 20% L-arabinose (0.2% final) (**step 3** in Subheading 3.2.1).
3. During opsonization (Fig. 2b), prepare two aliquots of infection medium containing 50 μg/mL gentamicin (G50) and 10 μg/mL gentamicin (G10), respectively (**step 6** in Subheading 3.2.1).
4. For the procedure described in **step 11** in Subheading 3.2.1:
   (a) Replace the medium with G50 and incubate plates at 37 °C in a 5% CO_2 incubator for 30 min.
   (b) While incubating collect the T0 time point as described in **step 13** in Subheading 3.2.1. Alternatively to PBS, bacteria can be resuspended in 1 mL of PBS containing 3% PFA if sample fixation is required prior to analysis. If PFA is used, incubate 10 min at RT, centrifuge at 17,000 × *g* for 2 min and resuspend in 800 μL of PBS. Then, transfer into FACS tubes with a cell strainer cap and keep at 4 °C (*see* **Note 10**).
   (c) After incubation, aspirate the medium and replace with G10.
5. Incubate between 10 and 18 h (**step 12** in Subheading 3.2.1) (*see* **Note 11**).
6. Following **step 13** in Subheading 3.2.1, transfer the 1 mL PBS suspensions into FACS tubes with a cell strainer cap and keep at 4 °C.

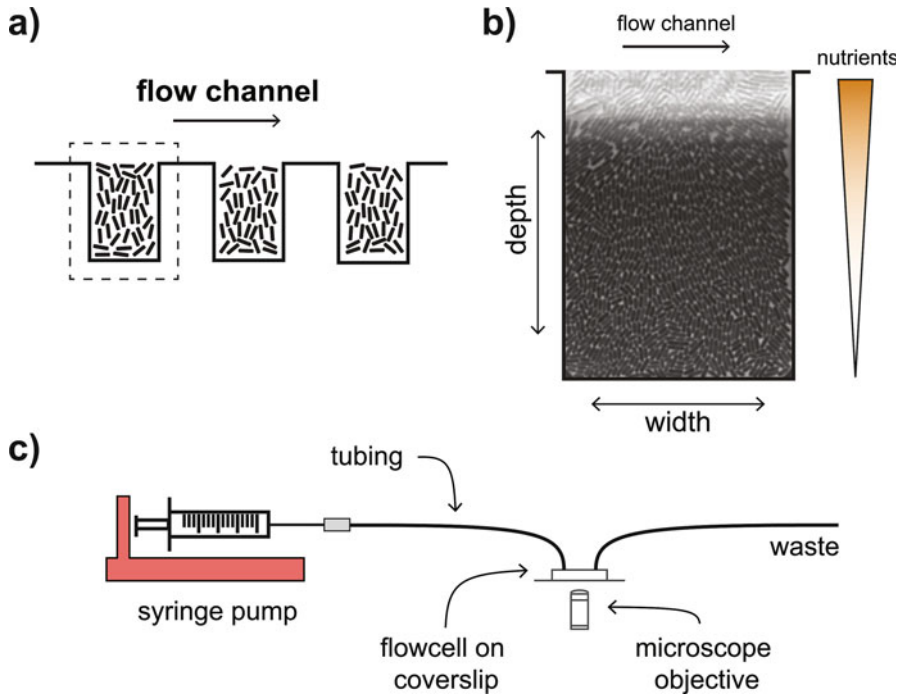

Fig. 2 Fluorescence dilution allows the visualization of growth rate heterogeneity in a clonal population during infection. Survival enumeration assays (Subheading 3.2.2) can lead to confusion between survival due to tolerance of the whole population and persistence of a subpopulation. We developed fluorescence dilution [3, 9, 10] to quantify the proportion of non-growing (NG) bacteria. (**a**) One fluorescence dilution reporter is the pFCcGi plasmid that encodes *mCherry* under the constitutive *rpsM* promoter (as a constitutive marker to track bacteria by flow cytometry and distinguish them from debris following release from macrophages) and *gfp* under the pBAD arabinose-inducible promoter (available at https:/www.addgene.org/59324/). (**b**) Preincubation of strains carrying pFCcGi with arabinose leads to expression of both mCherry and GFP in the whole population. Subsequently, removal of arabinose in new medium leads to dilution of preloaded GFP in bacteria and results in halving of GFP content at each division for growing bacteria (G). In comparison, non-growing bacteria (NG) retain high GFP levels. All bacteria remain mCherry positive throughout the experiment. (**c**) Comparison between the wild-type strain and a mutant that displays a high persistence phenotype in macrophages and higher proportions of NG as quantified by flow cytometry. It is noteworthy that NG are almost undetectable in the wild type at T24 due to the increased proportion of growing bacteria over time. *ON* overnight culture, *Ops.* opsonization, *BP* before phagocytosis, *Phago.* phagocytosis. *NG* non-growers, *G* growers, *WT* wild type

7. Analyze samples on a flow cytometer equipped to detect GFP (max excitation 488 nm, max emission 510 nm) and mCherry (max excitation 587 nm, max emission 610 nm) such as an LSR Fortessa flow cytometer (Beckton Dickinson) using FACSDiva software (Beckton Dickinson). Gate using FSC/SSC parameters as defined by a pure bacterial suspension. Then, to separate bacteria from unwanted debris, use the constitutive mCherry fluorescence as a positive marker for all bacteria.
8. Analyze the data using flow cytometry analysis software such as FlowJo (TreeStar, Inc.) considering these recommendations.

(a) The non-growing (NG) population displays the same GFP and mCherry intensities as the whole population at T0 while growing (G) bacteria have separated from the NG cells in diluting out their GFP signal (Fig. 2c). Relative proportions of growers and NG can therefore be easily deduced.

(b) Since the proportion of NG bacteria is a percentage of the total population, the extent of proliferation of the growing population influences it. To circumvent the potential bias in comparing strains with different growth rates, it is important to normalize data to the number of generations displayed by growers of each strain. To do so, the ratio of the geometric mean of GFP fluorescence of (1) the whole population at T0 and of (2) the growing population at T10 is calculated (Fig. 2c), giving the dilution factor F as indicated by the following formula:

$$F = \Upsilon_{T0}/\Upsilon_{T10}$$

where F is the dilution factor (referred to previously as "fold replication"), Υ_{T0} is the GFP fluorescence geomean of the population at T0, Υ_{T10} is the GFP fluorescence geomean of the growing population at T10.

(c) Since bacteria halve their GFP signal at each division, $\log_2(F)$ represents the number of generations N at a specific time, where $N = \log_2(F)$ (*see* **Note 12**).

3.2.4 Distinction Between Active and Inactive Non-growers (Fig. 3a)

Proceed with the infection of BMM as described in Subheading 3.2.1 following these modifications:

1. When seeding BMM, prepare a different plate for collection of each time point, with three technical replicates per strain per time point. For example, if a strain is compared to the wild type, two 6-well plates (T24 and T26) should be prepared with 6 wells each (*see* **Notes 6–8**).
2. Grow bacteria containing the pFCcGi plasmid in 10 mL of LB medium supplemented with 5 μL of 100 mg/mL carbenicillin (50 μg/mL final) but without arabinose (**step 3** in Subheading 3.2.1).
3. During opsonization, prepare infection medium containing 100 μg/mL cefotaxime (C100) (**step 6** in Subheading 3.2.1).
4. Incubate plates at 37 °C in a 5% CO_2 incubator for 24 h (**step 12** in Subheading 3.2.1).
5. At T24, replace the medium with prewarmed infection medium C100 0.2% arabinose and incubate at 37 °C in a 5% CO_2 incubator for 2 h. In parallel, collect BMM from the T24

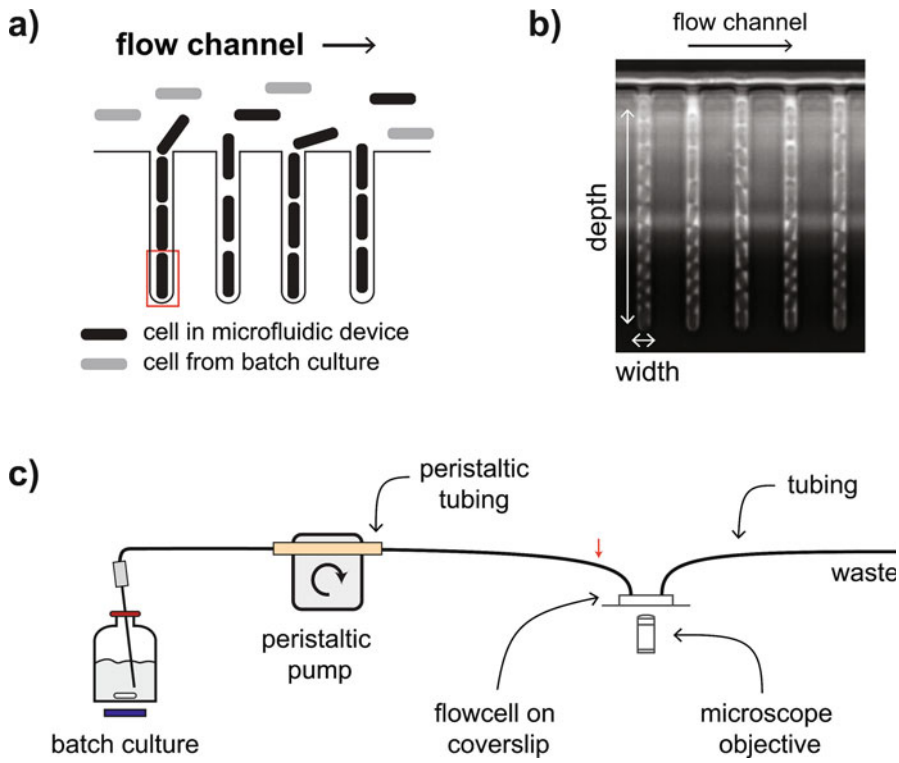

Fig. 3 Fluorescence accumulation allows the distinction between active and inactive non-growers. (**a**) The pFCcGi plasmid can be used to distinguish non-growers which are still able to produce GFP following the addition of the arabinose inducer to the infection medium. (**b**) Addition of arabinose triggers the production of GFP by approximatively half of the non-growing bacteria that survive 24 h cefotaxime exposure in macrophages. *ON* overnight culture, *Ops.* opsonization, *BP* before phagocytosis, *Phago.* phagocytosis, *NG* non-growers, *iNG* inactive non-growers, *aNG* active non-growers

plate as described in **step 13** in Subheading 3.2.1. Alternatively to PBS, bacteria can be resuspended in 1 mL of PBS containing 3% PFA if samples fixation is required prior to analysis by flow cytometry. If PFA is used, incubate 10 min at RT, centrifuge at 17,000 × *g* for 2 min and resuspend in 800 µL of PBS. Then, transfer into FACS tubes with a cell strainer cap and keep at 4 °C (*see* **Note 10**). At T26, collect BMM from the T26 plates as described for T24 (*see* **Note 13**).

6. Analyze T24 and T26 samples on a flow cytometer equipped to detect GFP (max excitation 488 nm, max emission 510 nm) and mCherry (max excitation 587 nm, max emission 610 nm) such as an LSR Fortessa flow cytometer (Beckton Dickinson) using FACSDiva software (Beckton Dickinson). Gate using FSC/SSC parameters as defined by a pure bacterial suspension. Then, to separate bacteria from unwanted debris, use the constitutive mCherry fluorescence as a positive marker for all bacteria.

7. Distinction between active and inactive non-growers is based upon accumulation of GFP induced upon addition of arabinose. Thus, active non-growers form a distinct population exhibiting a higher GFP signal than the inactive non-growers (Fig. 3b). This population can be expressed as a percentage of the total NG population. In addition, the sorted population of active-non growers (aNG) can be used for a wide range of applications, including high-throughput methods (described in Subheading 3.2.5).

3.2.5 Overview of Bacterial Persistence Using Dual RNAseq

The following section describes in detail the protocol used to obtain total RNA from the different populations shown in Fig. 4. The later steps such as ribosomal depletion, cDNA preparation, RNA-sequencing and data analysis are also described in brief. References have been added to help the reader find protocols and guidance for each of these steps, from cDNA library preparation to data analysis.

Proceed with the infection of BMM as described in Subheading 3.2.1 following these modifications:

1. When seeding BMM, seed fresh BMM onto seven 6-well plates in infection medium (1×10^6 BMM/well) (*see* **Note 14**).
2. Grow bacteria containing the pFCcGi plasmid in 50 mL sterile plastic tubes containing 10 mL of MgMES medium supplemented with 5 μL of 100 mg/mL carbenicillin (50 μg/mL final) and 100 μL of 20% L-arabinose (0.2% final) ON (16 h, OD_{600} around 0.8) (**step 3** in Subheading 3.2.1).
3. Opsonize the ON cultures for 20 min at RT by mixing together 180 μL of prewarmed infection medium, 80 μL of ON bacterial suspension and 20 μL of mouse serum (**step 5** in Subheading 3.2.1).
4. During opsonization, prepare two aliquots of infection medium containing 50 μg/mL gentamicin (G50) and 10 μg/mL gentamicin (G10), respectively (**step 6** in Subheading 3.2.1).
5. Infect each BMM well with 200 μL of opsonized bacteria to have an MOI around 10 (**step 8** in Subheading 3.2.1) (*see* **Note 15**). In total, six 6-well plates should be infected. The remaining uninfected plate should be collected immediately.
6. Aspirate the medium and replace it with G50 prewarmed medium (T0 of the infection) (**step 11** in Subheading 3.2.1). After 30 min of incubation at 37 °C in a 5% CO_2 incubator, aspirate G50 and replace it with prewarmed G10. Leave the plates for another 17.5 h in the incubator before collection.
7. Extract total RNA from the inoculum (ON bacteria in stationary phase used to infect macrophages), for example, using the

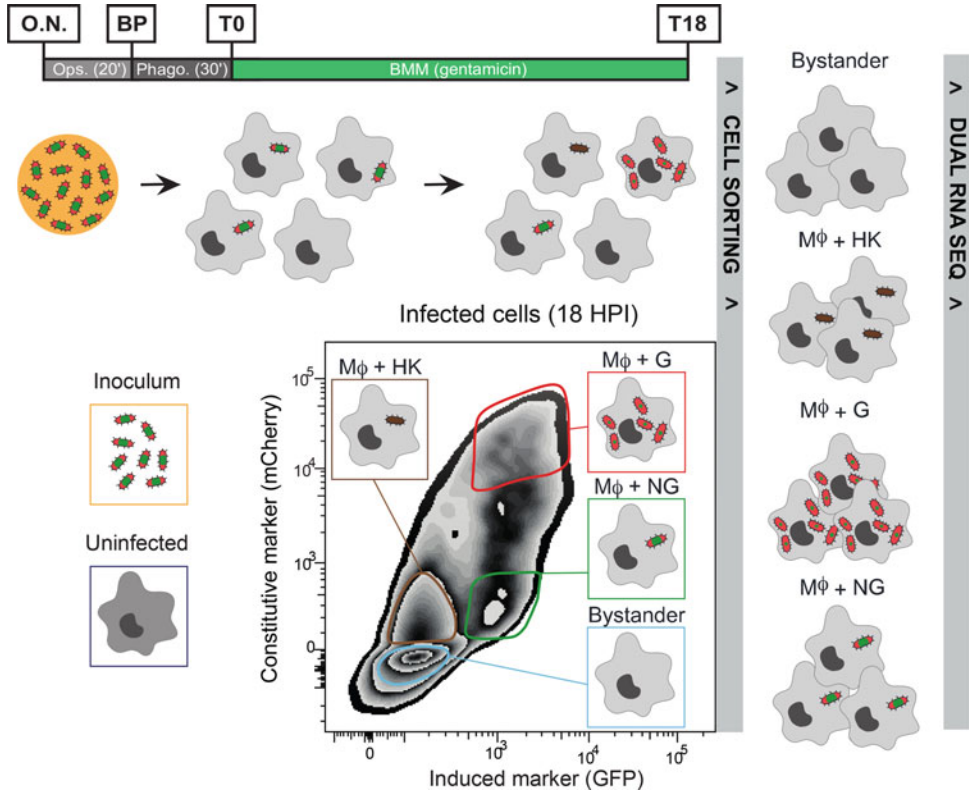

Fig. 4 Different populations on which dual RNAseq can be performed. DualRNAseq can be performed on three different categories of cell populations: (1) the bacterial inoculum only; (2) naïve (uninfected) macrophages only; and (3) macrophages 18 h post-infection. This third category can be subdivided further into four subpopulations defined by the fluorescence profile of the macrophages. These are bystander macrophages or macrophages that contain host-killed (HK), growing (G) or non-growing (NG) bacteria. The GFP signal is used to resolve bystander macrophages from those that were infected, in that infected macrophages emit one unit of GFP signal (the initial GFP load is retained by a non-growing bacterium, or divided between progeny of growing bacteria within the same host cell). The mCherry signal is used to resolve growing from non-growing bacteria, in that its constitutive expression causes accumulation of red fluorescent signal as the bacterial biomass increases within the same macrophage. The signal from both fluorophores is used to discern macrophages that killed the bacterium following phagocytosis, as here the GFP signal is bleached yet the mCherry signal is retained long enough to be detected. Exploiting these different profiles facilitates cell sorting and subsequent sequencing

mirVana kit (Thermofisher Scientific). All solutions referred to in subsequent steps are present in the kit (*see* **Note 16**).

8. To collect BMM (**step 13** in Subheading 3.2.1), aspirate the infection medium, add 500 µL of ice-cold PBS onto each well and gently scrape off the macrophages. Collect the uninfected and the pooled infected wells in two separate FACS tubes with cell strainer caps. Flow cytometry is then used to discriminate the different subsets of macrophages, as described in **Note 17**.

9. Sort unfixed samples of uninfected macrophages (to obtain the naïve samples) and infected macrophages (to obtain the bystander, the host-killed, the non-growers and the growers samples) under continuous cooling at 4 °C using a cell sorter such as the BD FACS Aria III (Fig. 4) (*see* **Note 17**). Sort 10^5 events of each subpopulation.
10. Extract RNA of each subpopulation according to the following steps:
    (a) Isolate total RNA using the mirVana kit.
    (b) Store the RNA eluate at −20 °C.
    (c) Measure RNA concentrations with a NanoDrop using 1 μL of each sample.
    (d) Treat RNA eluates with DNase I. Perform the reaction in 50 μL total with 5 μL of 10xDNase I buffer + $MgCl_2$, 0.25 U of DNase I per μg of RNA, and RNase inhibitor such as 0.5 μL of SUPERaseIN. Incubate the mix for 45 min at 37 °C.
    (e) Put the tubes on ice and add 100 μL of nuclease-free dH_2O.
    (f) Transfer the mix into a phase-lock tube and add 150 μL of RotiAqua PCI (phenol, chloroform and isoamyl alcohol at a ratio of 25:24:1, pH 4.5–5).
    (g) Mix vigorously and centrifuge at 17,000 × *g* for 15 min at 4 °C.
    (h) Transfer the upper phase (aqueous phase) into a new microfuge tube, make note of the volume and add 2.5 volume of precipitation mix (30:1 100% ethanol:3 M sodium acetate pH 6.5).
    (i) Precipitate at −80 °C for 40 min, or at −20 °C for at least 3 h. Samples can be left at −20 °C overnight.
    (j) Centrifuge at 17,000 × *g* for 30 min at 4 °C.
    (k) Wash the RNA pellet using 80% ethanol.
    (l) Centrifuge at 17,000 × *g* for 10 min at 4 °C.
    (m) Discard the ethanol and air-dry the pellet which should be ethanol free before resuspension in nuclease-free dH_2O.
11. Deplete ribosomal RNA for each subpopulation by treating 100 ng DNase-treated total RNA with the Illumina Ribo-Zero Plus rRNA Depletion kit (*see* **Note 18**).
12. Prepare and sequence the cDNA libraries following the detailed protocol provided in [6] (*see* **Note 19**). This protocol provided by Vertis Biotechnologie AG is briefly summarized below:

(a) Fragment at least 100 ng of RNA by sonication (4 pulses of 30 s at 4 °C) in order to obtain sheared fragments between 200 and 400 bp.

(b) Dephosphorylate fragmented samples using Antarctic phosphatase and rephosphorylate using T4 polynucleotide kinase.

(c) Add poly(A)-tails to RNA using a poly(A) polymerase.

(d) Remove bacterial and eukaryotic 5′ cap using a pyrophosphatase enzyme (Cap-clip Acid pyrophosphatase—Cellscript) to liberate a 5′ monophosphate on each fragment for ligation of an RNA adaptor.

(e) Ligate 3′ adaptors containing a different barcode sequence for each sample.

(f) Synthesize the first-strand cDNA based on the 5′ and 3′ adaptors ligated to each RNA extremity using the M-MLV reverse transcriptase.

(g) Amplify the obtained cDNA using a high-fidelity DNA polymerase for a variable number of cycles to have sufficient amplification, typically 10–16. Overamplification will greatly decrease sample diversity. Based on the fragmentation, a signal on a TBE gel stained with SYBR Gold between 140 and 300 bp would be considered sufficient amplification whereas a signal greater than 300 bp would suggest overamplification.

(h) Purify the cDNA libraries using the Agencourt AMPure XP kit to remove excess primers and primer-dimers and analyze by Bioanalyzer.

(i) Assess the concentration of each cDNA library by either qPCR or Qubit measurement. Pool all libraries in equimolar amounts.

(j) Sequence the samples on the NextSeq500 platform (Illumina) using 75 cycles and single-end sequencing. A minimum of ten million reads per library should be aimed for (*see* **Note 20**).

(k) For each condition, biological triplicates should be sequenced and analyzed.

13. Process raw data. A full procedure on how to analyze the obtained raw data and examples of accurate downstream analysis are detailed in the supplementary material of [4].

4 Notes

1. Preincubation in fresh medium allows bacteria to exit stationary phase and resume growth. Consequently, growing cells are more susceptible to antibiotics in contrast with the highly tolerant bacteria from stationary phase [7]. It is noteworthy that differences in growth rates of different strains could affect the number of growing cells present when the antibiotic is added. Since the number of colony forming units (CFU) at T2, T4, and T6 are normalized to that at T0 for each strain, the preincubation period should be limited to 30 min to minimize the impact of any growth rate differences.
2. Even though appropriate dilutions should be adapted for each time point based on the strains used, the optimal dilutions with *Salmonella* Typhimurium 14028 for each time point are as follows: 10^{-5} at T0, 10^{-4} and 10^{-3} at T2, 10^{-3} and 10^{-2} at T4 and T6. If dilutions lower than 10^{-2} must be plated for a time point, there is a risk of transferring antibiotic from the culture to the LB plate. In this scenario, it might be necessary to add a washing step, usually done twice in LB.
3. It is important to continue the experiment for long enough with at least four time points to ensure the lower killing rate observed can be attributed to a persister population as opposed to a population-wide tolerance (described in [1]). In addition, fluorescence dilution (*see* Subheadings 3.2.3 and 3.2.4) can be done at the same time points in the absence of antibiotic to observe the proportion of non-growers in the whole population.
4. The protocol to prepare BMM is described in a prior version of this chapter [3].
5. Before infecting wells, check macrophages under a microscope to ensure of their adhesion and confluency.
6. It is important to have different plates for different time points to avoid keeping BMM out of the incubator until the time of collection.
7. Due to the intrinsic variability of the experiment itself, be sure to prepare at least two wells per strain/condition as technical duplicates. In addition, the assay should be repeated 3–5 times.
8. This protocol has been established to assess the number of persisters at a certain time point, here 24 h of infection. In vivo time course persister assays can also be done following the same protocol and covering more time points (for example, T0, T4, T8 and T24).
9. As described for the in vitro persister assay (Subheading 3.1), a single time point (here 24 h) does not allow discrimination

between antibiotic persistence and tolerance. Several time points can be assessed, or this experiment can be coupled with fluorescence dilution to avoid confusion between these two distinct phenomena (*see* Subheadings 3.2.3 and 3.2.4).

10. Fixed samples can be kept at 4 °C in the dark for a up to 5 days without affecting the quality of the fluorescent signal.
11. To ensure accurate quantification, it is extremely important to use a time point that allows a maximal separation between GFP fluorescence of the growing and the non-growing populations.
12. To avoid any misinterpretation, only the relative proportion of non-growers in strains that have an overall similar ability to proliferate should be compared. In addition, separation of the growing and the NG population is less accurate in mutants with proliferation defects since the GFP dilution rate is lower.
13. According to our results, the best time point to separate GFP-positive active (aNG) and GFP-negative inactive (iNG) non-growing bacteria is 2–4 h after addition of arabinose.
14. Seven plates are necessary: six for infecting and one for an uninfected control. At 18 h, infected cells are collected and the BMM are sorted by flow cytometry into four subpopulations based on their intracellular bacterial population. The subpopulations are: bystanders (BMM subjected to bacterial infection but without any bacterial phagocytosis), host-killed or HK (BMM that have killed internalized intracellular bacteria), non-growers or NG (BMM containing a non-growing bacterium) and growers or G (BMM containing growing bacteria). In addition, uninfected macrophages are also collected and constitute the naïve subpopulation.
15. The opsonization mix volumes have to be scaled up depending of the number of wells to infect.
16. Starting from the RNA extraction to the cDNA library procedures, all tubes and distilled water should be nuclease free. Filter tips are recommended.
17. Gates are set to only include macrophages in which a single phagocytosis event has taken place, based on the overall GFP intensity of the macrophages. Apoptotic BMM and doublets are excluded by gating.
18. Ribosomal depletion is performed strictly following manufacturer guidance provided by Illumina: https://support.illumina.com/downloads/truseq-stranded-total-rna-with-ribo-zero-plus-rrna-depletion-ref-guide.html
19. The construction of the cDNA libraries and RNA-sequencing can be done using the protocol described in this chapter. This protocol has been used in numerous seminal transcriptomic studies on *Salmonella*, notably [6, 8].

20. Sequencing for each library should aim to exceed ten million reads. However, to compensate for differences in bacterial genome coverage between samples with growing or non-growing bacteria, libraries derived from macrophages with non-growing bacteria should be sequenced three to five times deeper.

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References

1. Balaban NQ, Helaine S, Lewis K et al (2019) Definitions and guidelines for research on antibiotic persistence. Nat Rev Microbiol 17:441–448
2. Gollan B, Grabe G, Michaux C et al (2019) Bacterial persisters and infection: past, present, and progressing. Annu Rev Microbiol 73:359–385
3. Helaine S, Thompson JA, Watson KG et al (2010) Dynamics of intracellular bacterial replication at the single cell level. Proc Natl Acad Sci U S A 107:3746–3751
4. Stapels DAC, Hill PWS, Westermann AJ et al (2018) *Salmonella* persisters undermine host immune defenses during antibiotic treatment. Science 362:1156–1160
5. Fisher RA, Cheverton AM, Helaine S (2016) Analysis of macrophage-induced *Salmonella* persisters. In: Michiels J, Fauvart M (eds) Bacterial persistence: methods and protocols. Springer, New York, NY, pp 177–187
6. Westermann AJ, Förstner KU, Amman F et al (2016) Dual RNA-seq unveils noncoding RNA functions in host-pathogen interactions. Nature 529:496–501
7. Tuomanen E, Cozens R, Tosch W et al (1986) The rate of killing of *Escherichia coli* by beta-lactam antibiotics is strictly proportional to the rate of bacterial growth. J Gen Microbiol 132:1297–1304
8. Srikumar S, Kröger C, Hébrard M et al (2015) RNA-seq brings new insights to the intra-macrophage transcriptome of *Salmonella* Typhimurium. PLoS Pathog 11:e1005262
9. Figueira R, Watson KG, Holden DW et al (2013) Identification of *Salmonella* pathogenicity island-2 type III secretion system effectors involved in intramacrophage replication of *S. enterica* serovar Typhimurium: implications for rational vaccine design. mBio 4: e00065
10. Helaine S, Cheverton AM, Watson KG et al (2014) Internalization of *Salmonella* by macrophages induces formation of nonreplicating persisters. Science 343:204–208

INDEX

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Methods in Molecular Biology, vol. 2357, https://doi.org/10.1007/978-1-0716-1621-5,

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