Kim Lewis *Editor*

Persister Cells and Infectious Disease

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Editor
Kim Lewis
Antimicrobial Discovery Center
Northeastern University
Boston, Massachusetts, USA

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Introduction

This volume brings together leaders of the emerging field of persistence and antibiotic tolerance to present the state of the art and provide a roadmap for future studies. Drug-tolerant persisters form stochastically in bacterial populations, and were first described by Joseph Bigger in 1944. However, it took a long time for the importance of persisters to be recognized. This recognition is still a work in progress—for one, the attention of the scientific community and the public has been focused on the antimicrobial resistance (AMR) crisis we are currently experiencing. Antibiotic discovery lags behind the rapid acquisition and spread of resistance, and we now have pan-resistant pathogens such as *Acinetobacter baumannii*. Very considerable resources have been dedicated to fight AMR by governments and private Foundations. This is a subject that is commonly discussed at the UN and the WHO. After a long dry spell, we are finally seeing promising new lead compounds to treat AMR pathogens, such as teixobactin and arylomycin. At the same time, most infections are caused by drug-susceptible pathogens. Most patients in the hospital have challenging infections, which require lengthy treatment regimens, often with multiple antibiotics. The inability to rapidly eradicate a drug-susceptible pathogen is the main problem in the clinic. This problem stems from bacterial tolerance, the ability to survive a lethal dose of antibiotic, and is often associated with biofilms forming on indwelling devices and soft tissues. Persister cells confer tolerance to a population of bacteria in chronic infection.

The significant burden of chronic infections in the developed world is dwarfed by the global epidemic of tuberculosis. The disease requires an unusually lengthy treatment, and the consensus is that dormant cells are responsible for this. So far, the study of persisters, with a focus on conventional pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, happened very much in isolation from the work on drug-tolerant *Mycobacterium tuberculosis*. This volume for the first time brings these two fields together—we have two chapters on *M. tuberculosis* drug tolerance. This should really be a single field, and we hope that this volume will serve as a link for researchers working on the same problem with different pathogens.

While we have a good understanding of the mechanisms of antibiotic resistance, this is not the case with antibiotic tolerance. Similarly, and perhaps not surprisingly, approaches to eradicate persisters are lagging. Once the AMR crisis is behind us, we will still be facing the daunting task of combatting persister cells.

The relatively slow pace in the study of persisters has been not only due to the late realization of their important role in chronic infections but also due to objective difficulties in studying a small subpopulation of cells with a fleeting phenotype. Advanced tools for the study of single cells have become available, and several chapters in this book describe experiments with persisters using cell sorting, microfluidics, and microscopy, in addition to traditional molecular and biochemical approaches. Studies of persister formation point to two types of mechanisms—specialized and general. Toxin/antitoxin modules represent the specialized mechanisms operating under particular conditions, while relative dormancy, with metabolic inactivity and low ATP, is emerging as a possible general mechanism of persister formation.

This volume also covers early advances in the discovery of anti-persister compounds. The mechanisms of action of the first anti-persister compounds provide a blueprint for additional discoveries, and are a cause for optimism in achieving the ultimate goal of developing sterilizing antibiotics.

This is an exciting time to be joining the field of persister studies—the tools have been developed, the knowledge base has been established, but the big discoveries are still waiting in the wings.

Boston, MA Kim Lewis

Contents

Contributors

Robert B. Abramovitch Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI, USA

Nathalie Q. Balaban Racah Institute of Physics, The Hebrew University, Jerusalem, Israel

Bork A. Berghoff Institute for Microbiology and Molecular Biology, Justus Liebig University Giessen, Giessen, Germany

Maikel Boot Department of Microbial Pathogenesis, Yale University, New Haven, CT, USA

Mark P. Brynildsen Department of Chemical and Biological Engineering, Princeton University, Princeton, NJ, USA

Iliana Escobar Division of Infectious Diseases, Rhode Island Hospital, Department of Medicine, Warren Alpert Medical School of Brown University, Providence, RI, USA

Nathan Fraikin Cellular and Molecular Microbiology, Faculté des Sciences, Université Libre de Bruxelles (ULB), Gosselies, Belgium

Beth Burgwyn Fuchs Division of Infectious Diseases, Rhode Island Hospital, Department of Medicine, Warren Alpert Medical School of Brown University, Providence, RI, USA

Frédéric Goormaghtigh Cellular and Molecular Microbiology, Faculté des Sciences, Université Libre de Bruxelles (ULB), Gosselies, Belgium

Alexander Harms Biozentrum, University of Basel, Basel, Switzerland

Sophie Helaine Department of Medicine, MRC CMBI Imperial College London, London, UK

Pauline Herpels VIB, Center for Microbiology, Leuven, Belgium
KU Leuven, Centre of Microbial and Plant Genetics, Leuven, Belgium

Peter W. S. Hill Department of Medicine, MRC CMBI Imperial College London, London, UK

Arvi Jõers Insitute of Technology, University of Tartu, Tartu, Estonia

Niilo Kaldalu Insitute of Technology, University of Tartu, Tartu, Estonia

Wooseong Kim Division of Infectious Diseases, Rhode Island Hospital, Department of Medicine, Warren Alpert Medical School of Brown University, Providence, RI, USA

Kim Lewis Antimicrobial Discovery Center, Department of Biology, Northeastern University, Boston, MA, USA

Jiafeng Liu Racah Institute of Physics, The Hebrew University, Jerusalem, Israel

Hannes Luidalepp Quretec OÜ, Tartu, Estonia

Sylvie Manuse Antimicrobial Discovery Center, Department of Biology, Northeastern University, Boston, MA, USA

Jan Michiels VIB, Center for Microbiology, Leuven, Belgium
KU Leuven, Centre of Microbial and Plant Genetics, Leuven, Belgium

Wendy W. K. Mok Department of Chemical and Biological Engineering, Princeton University, Princeton, NJ, USA
Department of Molecular Biology and Biophysics, UConn Health, Farmington, CT, USA

Eleftherios Mylonakis Division of Infectious Diseases, Rhode Island Hospital, Department of Medicine, Warren Alpert Medical School of Brown University, Providence, RI, USA

Marta Putrinš Insitute of Technology, University of Tartu, Tartu, Estonia

Hesper Rego Department of Microbial Pathogenesis, Yale University, New Haven, CT, USA

Tanel Tenson Insitute of Technology, University of Tartu, Tartu, Estonia

Laurence Van Melderen Cellular and Molecular Microbiology, Faculté des Sciences, Université Libre de Bruxelles (ULB), Gosselies, Belgium

Natalie Verstraeten VIB, Center for Microbiology, Leuven, Belgium
KU Leuven, Centre of Microbial and Plant Genetics, Leuven, Belgium

E. Gerhart H. Wagner Department of Cell and Molecular Biology, Biomedical Center, Uppsala University, Uppsala, Sweden

Dorien Wilmaerts VIB, Center for Microbiology, Leuven, Belgium
KU Leuven, Centre of Microbial and Plant Genetics, Leuven, Belgium

Huiqing Zheng Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI, USA

Chapter 1
Evolution Under Antibiotic Treatments: Interplay Between Antibiotic Persistence, Tolerance, and Resistance

Nathalie Q. Balaban and Jiafeng Liu

Abstract In this chapter, we describe the experimental evolution of antibiotic tolerance and persistence under antibiotic treatments and how these phenomena can speed up the subsequent evolution of resistance. The first two parts are dedicated to defining the difference between antibiotic resistance, tolerance, and persistence with qualitative definitions and quantitative metrics. The third part describes experimental observations of the evolution of tolerance and persistence under antibiotic treatments. The fourth part shows that tolerance and persistence speed up the evolution of antibiotic resistance. In each part, mathematical subsections can be skipped by the reader without losing the qualitative understanding of the effects.

1.1 Distinguishing Between Resistance, Tolerance, and Antibiotic Persistence

Following our recent works (Balaban et al. 2019; Brauner et al. 2016), we briefly characterize below antibiotic resistance, tolerance, and persistence. Within the antibiotic persistence phenotype, three main archetypes have been observed in vitro. We outline these archetypes in Sect. 1.1.4 and describe in Sect. 1.2 the differences in the experimental protocols required to measure the persistence levels for each type. We note that these definitions do not preclude the existence of other types of antibiotic persistence, but we chose to focus on those characterized already in several labs. Finally, we briefly describe a phenomenological mathematical model that allows identifying parameters that vary among different modes of survival.

N. Q. Balaban (✉) · J. Liu
Racah Institute of Physics, The Hebrew University, Jerusalem, Israel
e-mail: nathalie.balaban@mail.huji.ac.il

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1.1.1 Antibiotic Resistance

"Antibiotic resistance" is the *inherited* ability of bacteria to reproduce consecutively in the presence of a drug that would otherwise prevent the growth. The most widespread measure of the level of resistance is the Minimum Inhibitory Concentration (MIC) of the antibiotic, which prevents the replication of the bacteria. Higher resistance points to a higher MIC (Fig. 1.1a). Resistance is largely acquired by horizontal transfer of resistance gene cassettes (e.g., antibiotic inactivating enzymes (Jacoby 2009) or efflux pumps (Du et al. 2018)) or de novo mutations (e.g., altering the antibiotic target or reducing the uptake of antibiotics through the membrane (Blair et al. 2015)). Importantly, all these mechanisms result in a lower effective antibiotic concentration.

1.1.2 Antibiotic Tolerance

"Tolerance" is a transient ability of an *entire* population of bacteria to survive a bactericidal antibiotic treatment, without a change in the MIC, by slowing down a process that is required for antibiotic activity. Often, this slowing down also results in significantly slower growth and even growth arrest. The survival advantage of tolerant bacteria is often seen in treatments by drugs belonging to different classes,

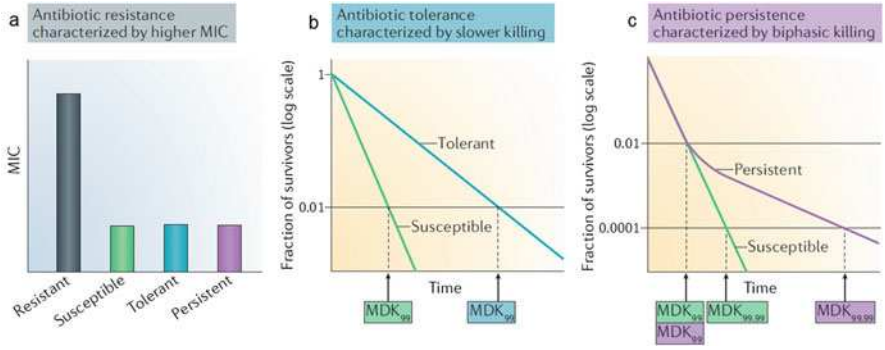

Fig. 1.1 Antibiotic resistance, tolerance, and persistence are distinct responses to antibiotic treatment that lead to increased survival compared with susceptible cells. (**a**) Resistant bacteria are characterized by a higher minimum inhibitory concentration (MIC). Antibiotic persistence and tolerance do not lead to an increase in the MIC compared with susceptible bacteria. (**b**) Tolerance is characterized by an increase in the minimum duration for killing [MDK; e.g., for 99% of bacterial cells in the population (MDK_{99})] compared with susceptible bacteria. (**c**) Persistence is a heterogeneous response of the bacterial population with a population of susceptible bacteria and a subpopulation of tolerant bacteria. Therefore, the MIC is the same as for susceptible bacteria and the MDK is different, depending on the subpopulation size. Here, a subpopulation of ~1% of tolerant bacteria leaves the MDK_{99} unchanged but affects the $MDK_{99.99}$. Adapted with permission from Balaban et al. (2019). Springer Nature Limited (this material is excluded from the CC-BY-4.0 license)

for example, β-lactams and fluoroquinolones (Wolfson et al. 1990), and even phages (Pearl et al. 2008). However, strains highly tolerant to these antibiotics may still be killed efficiently by other drugs. Although their survival under the antibiotic treatment to which they are tolerant is much higher than in non-tolerant strains, their MIC is unchanged (Fig. 1.1a), and another measure is introduced to characterize their slower killing: the Minimum Duration of Killing 99% of the bacterial population (MDK_{99}) (Fridman et al. 2014) (Fig. 1.1b).

1.1.3 Antibiotic Persistence

"Antibiotic persistence" (henceforth termed simply "persistence") enables a *subpopulation* of tolerant bacteria to survive in the presence of a bactericidal antibiotic. Persistent cells re-cultured on the fresh medium will demonstrate the same susceptibility to the same antibiotic as the initial culture, that is, only a subpopulation of the new culture will exhibit the persistent phenotype.

Unlike resistant cells, persisters cannot replicate in the presence of the drug any better than other cells but are killed at a lower rate than the susceptible population from which they are derived. This feature distinguishes persistence from heteroresistance, a phenomenon in which a small subpopulation transiently displays a substantially (>8-fold) higher MIC (El-Halfawy and Valvano 2015).

The hallmark of antibiotic persistence is the biphasic killing curve during the time-kill assays (Fig. 1.1c). On this curve, persisters correspond to the significantly slower killing phase after the bulk of the bacterial population is eliminated during the first phase of rapid killing.

Antibiotic tolerance and persistence are similar epigenetic traits that enable bacterial survival in the presence of bactericidal drugs. In some qualitative studies, the two terms may be interchangeable (Meylan et al. 2018). Nonetheless, differences do exist between persistence and tolerance. Persisters basically represent a *subpopulation* (typically <1%) of tolerant bacteria (thus, the phenomenon could have been called "heterotolerance") that can survive drug concentrations much higher than the MIC. Not surprisingly, mechanisms responsible for tolerance, such as dormancy, reduced metabolism, and ATP levels, have also been identified for persistence (Lewis 2007). What differentiates tolerance from persistence is the heterogeneous killing seen in the latter, that is, not all bacteria in a clonal culture are killed at the same rate. A subpopulation of persister cells is able to survive much better the antibiotic treatment than the majority of the population, as attested by the biphasic killing curve. Antibiotic persistence is not restricted to just two subpopulations. In the general case, more than one persister subpopulation may coexist and, thus, a multimodal killing curve is observed (Balaban et al. 2004). When studying persistence, two aspects are particularly interesting, the first one being pertaining to tolerance, and the second is specific to persistence: (1) the molecular mechanism(s) that enables tolerant bacteria to survive, and (2) the mathematical principle that generates heterogeneity in the population (Ackermann 2015), for example, nonlinear mechanisms leading to bimodality by amplifying stochasticity (Tsimring 2014; Huang et al. 2018).

1.1.4 Different Types of Persistent Bacteria

It is still a subject of hot debates whether a single general or multiple specific molecular mechanisms underlie the persistence phenotype (Levin et al. 2014; Michiels et al. 2016; Radzikowski et al. 2017), and the reader is referred to other chapters of this book. However, major mechanistically distinct ways for generating persisters in a culture have been identified. Distinguishing between the types of persistence is crucial, for each type requires a different procedure to measure the persistence level.

1.1.4.1 Triggered Persistence [Previously Called Type I (Balaban et al. 2004)]

In most observed cases, antibiotic persistence in bacteria is induced by external conditions, the commonest one being starvation. Even when the pressure is removed, for example, by diluting a starved overnight culture in fresh medium, some cells may still remain in the dormant state for extensive periods of time and may be found in the survival fraction. Even when the culture is regrown for a few hours and reaches what seems to be "exponential growth," a fraction of the persisters triggered by starvation may still be in a lag phase. Therefore, the lag time distribution of individual cells after exposure to a stress is an important factor that may determine the persistence level (Jõers et al. 2010; Levin-Reisman et al. 2010).

Numerous stress conditions have been identified to induce triggered persistence, among them starvation for various nutrients (Gutierrez et al. 2017), cell number (Vega et al. 2012), oxidative and acid stress, subinhibitory concentrations of drugs, immune factors, and exposure to immune cells (Helaine et al. 2014).

A further complication of the phenomenon is associated with high concentrations (Eagle and Musselman 1948) of antibiotics that trigger growth arrest, and cause a paradoxical lower killing rate and *drug-induced persistence* (Dörr et al. 2010). In this scenario, a bactericidal antibiotic becomes bacteriostatic for a subpopulation of cells that respond to the antibiotic signal itself, for example, by activating a stress response that enables them to survive (Dörr et al. 2010; Audrain et al. 2013). This type of response does not depend on the history of the culture prior to exposure to the drug (Johnson et al. 2013), and, therefore, may be attributed to spontaneous persistence. However, it may be more specific to the applied antibiotic and its concentration compared to other forms of persistence.

1.1.4.2 Spontaneous Persistence [Previously Called Type II (Balaban et al. 2004)]

Persistence may occur spontaneously in a steady exponentially growing culture. This form of persistence seems to be significantly less common than Type I persistence, and at present, no direct observations of spontaneous persistence have been clearly reported at the single-cell level in wild-type strains.

1.2 Quantification of Antibiotic Tolerance and Persistence

Tolerance is poorly characterized due to the lack of a quantitative and easily measured indicator similar to the MIC. The MDK_{99}—the minimum duration for killing 99% of the bacteria—can be defined when the killing rate reaches saturation at high concentration, for example, in Eq. (1.2) (Fridman et al. 2014; Brauner et al. 2017) (Fig. 1.1b). The MDK_{99} can be deduced from kill curves measured under antibiotic concentrations above the saturation regime (Brauner et al. 2017).

For characterizing the persistence of a bacterial population, a similar indicator such as $MDK_{99.99}$, can be used (Fig. 1.1c). However, if the persistence level itself is needed, the fraction of the tolerant subpopulation (α in Eq. 1.3) should be measured by extrapolating to slower killing curve to the initial measurement.

Predictive models of the survival of microorganisms under bactericidal drugs show that the MIC metric is insufficient to characterize the behavior, although it is widely used (EUCAST 2019; Barry et al. 1999). Common phenomenological models for the dependence of the survival, S, versus the concentration, c, or duration of treatment, t, are the Zhi function (Zhi et al. 1986), or $E_{\max}$ or Hill models (Levin and Udekwu 2010). Within the framework of these models, the killing rate, ψ, is described by three main parameters, which represent distinct underlying physico-chemical mechanisms: (1) the MIC, (2) MDK_{99}, and (3) the Hill coefficient for the steepness of the concentration dependence, k.

$$S(c,t) = \mathrm{e}^{\psi t} \tag{1.1}$$

$$\psi(c) = \frac{\ln(0.01)}{MDK_{99}} \cdot \frac{1 - \left(\frac{c}{MIC}\right)^k}{\frac{\ln(0.01)}{\psi_{\max} \times MDK_{99}} - \left(\frac{c}{MIC}\right)^k} \tag{1.2}$$

This general function predicts how the concentration of the antibiotic and its duration will affect the growth or death of a strain with growth rate without antibiotic, $\psi_{\max}$. Note that the common notation of the model uses $\psi_{\min} = \frac{\ln(0.01)}{MDK_{99}}$.

In this model, resistance is defined as an increase in the MIC, whereas tolerance is defined as an increase in the MDK_{99}. So far, the parameters describe a uniform population. When the population is heterogeneous, at least one of the parameters is heterogeneous.

Heteroresistance means that a subpopulation(s) of cells have a higher MIC than most bacteria in the population. In typical reports of heteroresistance, it is also assumed that the heritability of the increased MIC is long enough to create detectable colonies (Nicoloff et al. 2019).

Antibiotic persistence (which in this context could have been called heterotolerance) means that a subpopulation(s) of cells have a higher MDK_{99} than the major portion of the population. If we assume that the fraction of persisters is α, then the survival can be presented as the sum of the survival of two subpopulations with different killing rates: