Author’s accepted manuscript

Chapter 3: The Biology of Persister Cells in *E. coli*

Alexander Harms

Biozentrum, University of Basel, Basel, Switzerland
email: alexander.harms@unibas.ch; telephone: 0041 61 2072129

Abstract

Bacterial persisters are dormant, antibiotic-tolerant cells that are phenotypic variants formed within a regularly growing, drug-susceptible population. They differ from genetically or phenotypically resistant cells in that their survival of antibiotic treatment is rooted in a dormant physiology and not in the obstruction of drug-target interactions. In this chapter I assembled a concise overview of the formation, survival, and evolution of persisters formed by the model organism *Escherichia coli*. Though the formation of persister cells has stochastic aspects, it is often induced by starvation or stress as a specialized differentiation of part of the population (“responsive diversification”). Consequently, the phenotypic heterogeneity of persisters and regularly growing cells is commonly interpreted as a bet-hedging strategy that ensures population survival under the threat of catastrophic events and that at the same time optimizes the benefit from favorable conditions. Multiple different molecular mechanisms have been implicated in persister cell formation and can be grouped into two major classes. Non-specific mechanisms affect bacterial physiology on a global scale via, e.g., alterations of energy metabolism, or are purely stochastic events that shut down cellular processes by accidental malfunctioning (“persistence as stuff happens”). Conversely, specialized mechanisms directly inhibit antibiotic targets often through activation of fine-tuned molecular switches known as toxin-antitoxin modules. In addition, the repair of cellular damage caused by antibiotics is critical for the resuscitation of persister cells. A major obstacle to coherently interpreting these findings is the fragmented nature of the literature and several controversies that should be consolidated by future studies.

Alexander Harms
Biozentrum, University of Basel
Basel, Switzerland
Email: alexander.harms@unibas.ch

Keywords

Antibiotic Tolerance; Bacterial Persister; Toxin-Antitoxin Module; Phenotypic Heterogeneity

3.1 Basic Concepts of Persister Cell Biology

The prevailing crisis of antibiotic therapy is often seen as a consequence of rising antibiotic resistance (Laxminarayan et al., 2013). However, chronic and relapsing infections are often associated with genetically susceptible bacteria that survive even massive and long-lasting antibiotic treatment (Levin and Rozen, 2006, Fauvart et al., 2011; see also Chap. 5). This phenomenon is commonly linked to the formation of specialized "persister" cells that are transiently tolerant to nominally lethal doses of antibiotics. Traditionally, the tolerance of these cells is seen as rooted in a dormant physiological state in which the targets of antibiotic drugs are inactive and can thus not be poisoned or corrupted by the treatment, enabling bacterial survival (Harms et al., 2016, Lewis, 2010, Wood et al., 2013). A major obstacle for the targeted development of effective treatment options against persister cells is the lack of a comprehensive understanding which molecular mechanisms and physiological changes truly underly their antibiotic tolerance. Furthermore, progress is hampered by the redundant and multifactorial nature of known persister mechanisms but also by the use of different model systems, methodologies, and definitions / understandings in the field (Balaban et al., 2013, Kaldalu et al., 2016).

In this chapter, I will focus on the by far most well studied model organism for research on persister cells, *Escherichia coli*, and try to draw a comprehensive picture of the biology of persister cells of this organism. To this end, I will summarize the physiological concepts underlying the stress tolerance of persister cells, the different molecular pathways driving this phenomenon, and how these shape the survival and growth of a bacterial population. While most data have been generated with the laboratory strain *E. coli* K-12 MG165, I will also highlight insight from environmental and clinical strains like different isolates of uropathogenic

E. coli (UPEC). Urinary tract infections are notorious for frequent relapse after treatment which is often seen as a consequence of persister cell formation by UPECs (Blango and Mulvey, 2010, Goneau et al., 2014). For a comprehensive view on persister cell biology involving other organisms or deeper insight into specific aspects, the reader is referred to the other chapters of this book or one of the recent review articles by different groups (Van den Bergh et al., 2017, Harms et al., 2016, Fisher et al., 2017, Lewis, 2010, Wood et al., 2013).

3.1.1 Persister Formation as a Phenotypic Switch into Dormancy

The general paradigm of bacterial persister formation in distinction from antibiotic resistance is that these cells attain transient antibiotic tolerance through a phenotypic switch into a dormant, slow- or non-growing state (Balaban et al., 2004). Put differently, the key difference between antibiotic resistance and drug tolerance is that resistance mechanisms impair the ability of the antibiotic to reach its target while persister cell formation comprises changes of bacterial physiology that interfere with the lethal effects of target poisoning (Keren et al., 2004b). Consistently, it is well established that the rate of antibiotic killing for a given set of bacteria is closely correlated to their growth rate and that the enforced shutdown of cellular activities through bacteriostatic drugs or ectopic expression of toxic proteins readily induces drug tolerance (Vazquez-Laslop et al., 2006, Claudi et al., 2014, Ocampo et al., 2014). Nevertheless, the majority of non-growing cells that arise without experimental intervention (e.g., in stationary phase) are not antibiotic-tolerant (Orman and Brynildsen, 2013, Dörr et al., 2009, Harms et al., 2017, Keren et al., 2004a), indicating that persisters "are not simply non-growing cells" but exhibit additional changes in their physiology that underly their survival and resuscitation (Lewis, 2005).

The question of how persister cells can reversibly enter such a dormant state and survive lethal doses of antibiotics is intimately connected to the question of how antibiotics kill. Two major different views on this topic are prevalent in the field. One side understands antibiotics as drugs that poison or corrupt bacterial targets in a way that they "disrupt key functions of their target such that the activity of the crippled enzyme or multicomponent machine becomes toxic and reduces viability"

(Cho et al., 2014). This view is commonly accepted by the majority of researchers and underlies the “dormancy model” of bacterial persistence in that persister formation involves the (selective of generalized) shutdown of cellular drug targets as the underlying reason of their tolerance as well as their lack of (significant) growth (Harms et al., 2016, Lewis, 2010, Wood et al., 2013). An alternative, yet not inherently incompatible, view highlights that the actual killing of bacterial cells would not be caused by damage due to poisoned drug targets but due to their secondary induction of “active death processes” (Yang et al., 2017). More precisely, the killing activities of the various different classes of bactericidal antibiotics are proposed to converge in the production of reactive oxygen species (ROS) through metabolic perturbations (Kohanski et al., 2010). This idea has been repeatedly disputed (Renggli et al., 2013, Keren et al., 2013, Liu and Imlay, 2013), but different variants of this theme are regularly invoked to explain phenotypes linked to bacterial persisters and drug tolerance.

3.1.2 Stochasticity and Heterogeneity of Persister Formation

Although persisters cells are often described to exhibit multidrug tolerance, multiple studies have shown that the far majority of persisters in a given experimental sample exhibit drug-specific tolerance (comprehensively reviewed by Van den Bergh et al., 2017). As an example, studies exploring persister cells formed by various environmental, pathogenic, and laboratory strains of *E. coli* found that tolerance levels varied vastly between isolates and that tolerance to different antibiotics was not relevantly correlated (Wiuff and Andersson, 2007, Hofsteenge et al., 2013, Stewart and Rozen, 2012, Goneau et al., 2014, Luidalepp et al., 2011). Nevertheless, these studies also found a small proportion of multidrug-tolerant cells. Two relevant conclusions can be drawn from these findings: First, it is unlikely that global cellular dormancy causing multitarget inactivation and multidrug tolerance plays a major role in *E. coli* persister formation in nature, though this phenotype can readily be evoked by genetic screens or selection for high persister levels in diverse bacteria (Michiels et al., 2016, Van den Bergh et al., 2016, Fridman et al., 2014). The reason for this discrepancy might be the significant population-wide cost of persister cell formation that could be higher for multi-

drug tolerance (see below). Second, it is clear that any laboratory culture with a seemingly homogeneous population of clonal bacteria harbors a wide variety of phenotypically different types of persister cells. Consequently, batch culture experiments must be interpreted with caution, particularly also because mutant phenotypes can be highly sensitive to seemingly small changes in culture conditions (Luidalepp et al., 2011).

In laboratory experiments during unconstrained growth of *E. coli* it is easily observed that tolerant persisters have formed prior to drug treatment as non- or slow-growing cells among an isogenic population of regularly growing cells (Balaban et al., 2004). From the view of the experimenter, these cells have emerged stochastically because they compose a seemingly stable subpopulation among identical peers. However, it is not clear to which extent persister cell formation is really induced by such a blind pacemaker. Repeated cycles of growth and dilution during exponential growth of *E. coli* progressively reduced the levels of persister cells, indicating that the majority of initially detected persisters had been carried over from stationary phase in the inoculum (Keren et al., 2004a, Orman and Brynildsen, 2013). Consistently, the fraction of *E. coli* persisters in a classical exponential growth time kill curve assay in LB medium is more or less proportional to the inoculum (Harms et al., 2017). These observations demonstrate that 1) the fraction of antibiotic-tolerant cells forming during exponential growth is very small and 2) actually too small to significantly affect the results of most studies, despite the common notion that experiments were performed in "exponential phase". According to the original definition of Balaban et al. (Balaban et al., 2004), most of the literature on stochastic persister formation therefore deals with "type I persisters" carried over from stationary phase and not with "type II persisters" forming during exponential growth. Knowledge about antibiotic-tolerant cells that form truly stochastically is therefore very limited. I speculate that many of them might arise accidentally by "persistence as stuff happens" (see below) or might not be persisters by common sense at all and instead exhibit phenotypic resistance (see below).

Conversely, it appears that persister cell formation has a significant deterministic component in the sense that various forms of sublethal stress induce formation of these cells but without causing full conversion of population into persisters (Dörr et al., 2009, Goneau et al., 2014, Mordukhova and Pan, 2014). Similarly, the level of persister cells generally increases in many bacteria throughout culture growth as nutrients be-

come exhausted and the bacteria progressively enter stationary phase (Dörr et al., 2009, Harms et al., 2017, Keren et al., 2004a). These phenomena are best explained as "responsive diversification" in the sense that the diversification of the clonal population into different phenotypes is induced by environmental factors like stress and starvation (Kotte et al., 2014). The resulting phenotypic heterogeneity enables the population as a whole to be pre-adapted to different environmental conditions while at the same time maximizing the benefits from the currently encountered one.

3.1.3 Biological Functions of Persister Cells

The evolutionary success of a bacterial population is determined by its ability to reproduce. At first glance it seems counter-intuitive that clonal bacterial populations would form persister cells that seem to be a drain of resources which could better be invested in population growth. However, this kind of phenotypic heterogeneity is ubiquitous among bacteria and was also described in eukaryotes from yeast to humans (comprehensively described by Van den Bergh et al., 2017). The phenomenon is commonly interpreted as a risk-spreading strategy that ensures survival of the genotype in the face of unpredictable and lethal threats that would wipe out the fast-growing phenotype(s), a concept known as "bet hedging" (Veening et al., 2008). Consistently, the level of persister cells does not only differ significantly between different strains or isolates of an organism (see above) but can readily evolve and adapt to different treatment regimes in the laboratory with higher levels of persister formation being selected for under more narrow treatment regimens and duration of dormancy matching the treatment time (Fridman et al., 2014, Michiels et al., 2016, Van den Bergh et al., 2016). These results are reminiscent of the observation that uropathogenic *E. coli* from recurrent infections display higher persister levels than other uropathogenic isolates and that *hipA7*, a known high-persister mutation, has been observed among uropathogenic *E. coli* (Goneau et al., 2014, Schumacher et al., 2015). Conversely, the formation of persister cells also seems to have fitness trade-offs with other bacterial traits (antagonistic pleiotropy) by compromising stationary phase survival and causing extended lag phases (Stepanyan et al., 2015). Beyond a baseline caused by cellular noise, it is therefore clear

that the formation of antibiotic-tolerant persister cells is a genetically evolved trait of bacteria under strong selection by environmental conditions and physiological constrains (see also Chap. 9).

3.2 Unraveling the Genetic Basis of Persister Formation

3.2.1 Conceptual Overview

Since the first description of persister cells by J. Bigger in *Staphylococcus aureus* in 1944 (Bigger, 1944) a lot of articles have been published on the molecular mechanisms of how they form and how they survive antibiotic treatment (comprehensively reviewed by Van den Bergh et al., 2017). The key conclusions from multiple different classical genetic screens, candidate-based approaches, and physiological studies have been that these mechanisms are highly redundant on the population level but can be grouped into two major branches: While some mechanisms cause antibiotic tolerance by globally modulating bacterial physiology and / or through stochastic events (non-specific mechanisms; see Sect. 3.3), others shut down one or more cellular functions via dedicated mechanisms (specialized mechanisms; see Sect. 3.4). In addition, different damage repair pathways contribute to the survival of persister cells (Sect. 3.5). The line between these groups is often blurred because also very targeted, specialized mechanisms can have consequences for global physiology, by intimate links between different persister mechanisms, and because a shutdown of cellular processes generally favors the repair of damage.

As far as they are not just stochastic, these molecular mechanisms are not controlled by specific "persister regulators" but rather integrated into the regulons of different arms of bacterial stress signaling. Most importantly, the starvation-induced second messenger (p)ppGpp is a key factor in the induction of many different persister mechanisms (Hauryliuk et al., 2015). Beyond (p)ppGpp, other signals related to stress (via sigma factor RpoS), metabolism (via cAMP/CRP), or oxidative damage (via OxyR) contribute to the signaling underlying persister formation (Amato et al.,

2013, Harms et al., 2016, Molina-Quiroz et al., 2016, Vega et al., 2012). Intriguingly, one dedicated study demonstrated that the type of persister cells formed during carbon source transitions of *E. coli* depends on the intracellular concentration of (p)ppGpp (Amato and Brynildsen, 2015; see also Chap. 7). Another important signaling pathway is the SOS response, a transcriptional program controlling various DNA repair functions, that is activated by single-stranded DNA arising as a consequence of DNA damage (Baharoglu and Mazel, 2014).

A major problem when summarizing the knowledge on different mechanisms of persister formation is that the methodologies used in the field to isolate and quantify persisters are highly variable and that the composition of the heterogeneous set of persisters is very sensitive to changes in experimental conditions. As an example, one article directly demonstrated that the qualitative observation and quantitative penetrance of different *E. coli* mutant phenotypes in persister formation was significantly influenced by the way of overnight culturing (Luidalepp et al., 2011). In consequence, the literature in the field can be described in the famous words of Fred Neidhardt as “apples, oranges, and unknown fruit” (Neidhardt, 2006). Many articles with seemingly contradictory results have therefore been published and debates about the validity of different studies are commonplace (Kaldalu et al., 2016, Goormaghtigh et al., 2018a, Harms et al., 2017, Van Melderen and Wood, 2017, Goormaghtigh et al., 2018b). It is therefore difficult to disentangle published results into a consistent picture of *E. coli* persister biology, so that in the following section I will merely summarize a number of important findings with the primary aim of illustrating key principles and concepts.

3.2.2 Distinguishing persister formation / survival from phenotypic resistance

A critical aspect when summarizing pathways of persister formation is that widely different conceptual frameworks of the terms “persister”, “tolerance”, etc. are used in the field (Van den Bergh et al., 2017, Balaban et al., 2013, Brauner et al., 2016). In this article I adhere to the classical view that persister cells are transient, phenotypic variants which are drug-tolerant in the sense that they survive nominally lethal antibiotic

concentrations killing their clonal peers. This antibiotic tolerance is rooted in a shutdown of drug targets through some kind of dormancy, either globally or selectively (Lewis, 2010, Harms et al., 2016, Wood et al., 2013). It is important to understand that this view does not comprise all imaginable mechanisms of how a cell can end up in the surviving subpopulation of a biphasic time-kill curve: Apart from classical persister cells, the survivors can merely exhibit phenotypic resistance (Corona and Martinez, 2013; see also Fig. 3.1 and Fig. 3.2). Key examples of this phenomenon are heterogenic expression of drug efflux pumps (Pu et al., 2016) or fluctuations in the expression of the multiple antibiotic resistance activator MarA (El Meouche et al., 2016) that have both been described in *E. coli*. In addition, a well-known example in mycobacteria are cells that by chance have expressed only low levels of KatG, the enzyme that activates the prodrug isoniazid, and can thus grow in presence of this antibiotic (Wakamoto et al., 2013). Consistently, a dedicated study using flow cytometry demonstrated that antibiotic survivors can form from regularly replicating bacteria, though quite rarely (Orman and Brynildsen, 2013). These different faces of single-cell phenotypic resistance are united in that they would, on the population level, increase the minimum inhibitory concentration (MIC) and enable bacterial growth in presence of the antibiotic. Phenotypic resistance is therefore fundamentally different from the tolerance exhibited by dormant, antibiotic-tolerant persister cells and requires different pharmacological strategies to be overcome. Consequently, I limit the content of this chapter to bacterial persister formation and survival in order to highlight the particular biology of this phenomenon.

3.3 Non-Specific Mechanisms of Persister Cell Formation

3.3.1 Energy Metabolism and Oxygen

Antibiotic tolerance and persister formation are intimately linked to cellular metabolism on multiple levels. Generally, it is commonly observed that the formation of persister cells is inversely correlated with metabolic activity and energy production, e.g., when evaluating different

bacterial mutants or growth conditions (Li and Zhang, 2007, Orman and Brynildsen, 2013, Shan et al., 2017). One core component of cellular energy metabolism is the electron transport chain (ETC), a series of protein complexes in the cytoplasmic membrane that transfers electrons from different donors like NADH or succinate onto receptors like oxygen. The energy generated along these electron transfers is used to pump protons out of the cytoplasm in order to create an electrochemical gradient known as the proton-motive force that fuels a variety of cellular processes including the synthesis of ATP. It is therefore unsurprising that *E. coli* strains carrying mutations in components of the ETC or its metabolic sources of electrons like GlpD or the TCA cycle displayed altered levels of persister formation (Ma et al., 2010, Spoering et al., 2006, Shan et al., 2015, Van den Bergh et al., 2016, Luidalepp et al., 2011). Intriguingly, these mutants showed phenotypes of both increased and decreased persister formation depending on gene, particular mutation, and experimental conditions (see note in Harms et al., 2016, and literature cited therein).

The mechanism of how the electron transport chain is closely linked to persister formation or survival has remained elusive. Under some circumstances the link is trivial because, e.g., the proton-motive force is critical for the uptake of aminoglycoside antibiotics (Krause et al., 2016). Therefore, providing specific metabolic stimuli to activate the ETC can dramatically ramp up the intracellular drug concentration in some persisters and kill them (Allison et al., 2011). A similar approach was also effective against fluoroquinolone-tolerant persisters, yet required to provide carbon source and terminal electron acceptor in order to globally restart respiratory metabolism (Gutierrez et al., 2017). Similarly, it is tempting to speculate that the ETC might affect persister formation or survival through its role in ATP production. Different groups have reported links between the antibiotic tolerance of persisters and low cellular ATP levels that could be caused by a controlled shutdown or random malfunctioning of the ETC (Wilmaerts et al., 2018, Shan et al., 2017).

Apart from these aspects of cellular metabolism, the ETC plays also a major role in the idea that antibiotics would kill bacteria through production of ROS. In this view (see above), the cellular effects of different antibiotic classes converge in a hyperactivation of the ETC as a source of superoxide ions that finally result in the production of hydroxyl radicals damaging cellular macromolecules (Kohanski et al., 2010). From this per-

spective, drug tolerance is primarily seen as a matter of interfering with this secondary killing activity of antibiotics, and persister phenotypes of different mutants are generally interpreted in this context (Yang et al., 2017). Several articles have indeed reported key roles of reducing oxidant stress and increasing ROS detoxification in *E. coli* persister survival dependent on (p)ppGpp or (the absence of) cAMP/CRP signaling (Molina-Quiroz et al., 2016, Nguyen et al., 2011).

3.3.2 PASH: "Persistence as Stuff Happens"

One major alternative theory to concepts based on the various genetically encoded pathways covered in this book is "PASH" or "Persistence As Stuff Happens" (Levin et al., 2014, Johnson and Levin, 2013, Vazquez-Laslop et al., 2006). In this view, the various types of heterogeneous persister cells primarily form more or less by chance as the "inadvertent consequence of different kinds of glitches and errors" in cellular metabolism and replication (Levin et al., 2014). It is largely undisputed that PASH exists and that it can well explain why the formation of persisters is ubiquitous among all organisms where it has been studied and why all attempts to create a mutant not forming persisters have failed (Johnson and Levin, 2013). As an example for clear PASH, accidents during DNA replication and other DNA processing functions result in activation of the SOS response in almost 1% of *E. coli* during unconstrained exponential growth (Pennington and Rosenberg, 2007). This phenomenon causes a heterogeneity of different states of SOS expression in the population and, in cells with very high SOS induction, will induce antibiotic tolerance through the different SOS-controlled pathways of persister formation or survival (see below and Fig. 3.2). It will be interesting to see future studies using single-cell approaches disentangling the molecular basis of different types of persister cells in a bacterial population and explore to what extent PASH is responsible for their formation and survival.

3.4 Specialized Mechanisms of Persister Cell Formation

Beyond broad changes in cellular physiology that interfere with antibiotic killing, persister cell formation has also been linked to a variety of more specific mechanisms that either shut down specific targets or are distinct from other more general mechanisms in that they act through fine-tuned molecular switches. Among these, toxin-antitoxin (TA) modules must be highlighted particularly. They are small genetic elements encoding a toxic protein and an antitoxin that can unleash this toxin’s activity in response to cellular signaling (Harms et al., 2018). TA modules exhibit two features that make them well-suited as effectors of persister cell formation, the ability to shut down cellular processes through toxin activation and a multilayered autoregulation that can control the bistability of persister formation as well as the entry into and, equally important, exit from the persister state. The links between TA modules and persisters will be covered in detail in Chap. 9 but are shortly outlined below.

3.4.1 Type I Toxin-Antitoxin Modules

Type I TA modules are defined by the RNA nature of the antitoxin and its control of toxin activity by regulating toxin translation (Berghoff and Wagner, 2017). They are the most well-established TA modules for persister formation of *E. coli* and two different representatives have been studied in details, the HokB/*sokB* system and the TisB/*istR* system (covered in detail in Chap. 6 and Chap. 8). Both have toxins that are small membrane-targeting peptides, but while HokB/*sokB* is controlled by second messenger (p)ppGpp and the elusive GTPase Obg, TisB/*istR* is controlled by DNA damage signaling via the SOS response (Dörr et al., 2010, Verstraeten et al., 2015). For both it has been unraveled how the interaction of RNA antitoxin and toxin mRNA as well as RNA structures and regulatory elements can control the frequency, inducibility, and duration of persistence (Berghoff and Wagner, 2017). The TisB and HokB toxins impair inner membrane integrity, causing membrane depolarization and shutting down many cellular processes either directly (if they are powered by the proton-motive force) or indirectly (through the resulting drop

in ATP levels; see also Fig. 3.2). Consequently, persister cells forming through HokB/*sokB* or TisB/*istR* activation are multidrug tolerant (Dörr et al., 2010, Verstraeten et al., 2015, Berghoff et al., 2017).

3.4.2 Type II Toxin-Antitoxin Modules

Until recently, type II TA modules were central to many discussions about *E. coli* persister formation because of debates about a prominently published pathway linking the two. In brief, this pathway was based on stochastic peaks of (p)ppGpp that would activate a specific set of these TA modules through stimulation of polyphosphate production (Maisonneuve and Gerdes, 2014, Van Melderen and Wood, 2017). In a major paradigm shift, it was recently demonstrated that this pathway does not exist and that the original studies supporting it suffered from a number of biological and technical shortcomings (Goormaghtigh et al., 2018a, Harms et al., 2017). A few additional articles reporting links between type II TA modules and persister formation of *E. coli* K-12 have been published, but these could often not be reproduced in other laboratories and did not have any follow-up studies (see articles by Goormaghtigh et al., 2018b, Van den Bergh et al., 2017, and literature cited therein). In the light of recent controversies, it seems therefore advisable to reserve a final conclusion on type II TA modules and persisters of *E. coli* K-12 for future studies that would approach the topic with an open mind and rigorous controls (see also Chap. 9). Regardless of the phenotypes of toxin-antitoxin deletions, gain-of-function mutants of type II TA modules have repeatedly been isolated in screens for bacterial mutants exhibiting high levels of persister formation (Moyed and Bertrand, 1983, Fridman et al., 2014). The most well-studied of these, *hipA7*, was also found among uropathogenic *E. coli* isolates (Schumacher et al., 2015). If TA modules are so easily converted into genetically encoded switches controlling persister formation, then it seems reasonable to speculate that at least some of these loci might be involved in this phenotype also in *E. coli* K-12 wildtype.

While research so far has mostly focused on roles of *E. coli* type II TA modules in stochastic persister formation, it would be interesting to follow up on previous work that had studied the activation of these TA

modules by different biologically relevant stresses (Shan et al., 2017). Intriguingly, type II TA modules of *Salmonella* Typhimurium are specifically essential for the strong induction of persister formation under starvation and acid stress after phagocytosis (Helaine et al., 2014).

3.4.3 Controlled inhibition of antibiotic target processes

β-lactam antibiotics cause futile cycles of the peptidoglycan biosynthesis machinery and thus poison cell wall formation (Cho et al., 2014). The killing rate of β-lactam treatment is therefore directly correlated with bacterial growth rate, and non-growing cells are inherently tolerant to these drugs (Lee et al., 2018). Consequently, any mechanism inhibiting bacterial growth will cause collateral tolerance specifically to β-lactam killing. In addition, a specific pathway has been described in which peaking (p)ppGpp levels shut down peptidoglycan biosynthesis in response to signaling involving ClpA and trans-translation (Amato and Brynildsen, 2015; see also Fig. 3.2).

Fluoroquinolone antibiotics poison DNA gyrase and topoisomerase IV, causing the formation of covalent complexes in which the enzyme-drug complex bridges a DNA double-strand break. This is principally reversible, but open DNA breaks can form upon collision of these "roadblocks" with replication forks and other DNA tracking systems, requiring DNA double-strand break repair (see below). Lethality arises through chromosome fragmentation when this system is overwhelmed (Aldred et al., 2014; see also below). Consequently, direct mechanisms of FQ tolerance need to ramp down DNA tracking systems and / or ramp up DNA repair functions. One elegant study demonstrated that *E. coli* can become fluoroquinolone-tolerant by modulating DNA gyrase activity through altered behavior of nucleoid-associated proteins in response to (p)ppGpp signaling (Amato et al., 2013, Amato and Brynildsen, 2015).

Aminoglycoside antibiotics are commonly thought to poison ribosomal translation by impairing the fidelity of the process in a way that mis-translation produces aberrant polypeptides causing cell damage (Krause et al., 2016). Consequently, direct mechanisms of aminoglycoside tolerance need to reduce translation rates and / or upregulate relevant cellular repair pathways. One study indeed implicated the ribosome mod-

ulation factor Rmf in the survival of *E. coli* under prolonged aminoglycoside treatment, indicating a role in persister formation or survival (McKay and Portnoy, 2015). Rmf is controlled by (p)ppGpp and can convert ribosomes into an inactive, dimeric conformation that has mostly been studied in the context of stationary phase biology (Gohara and Yap, 2018).

3.5 Repair of drug-related damage and persister resuscitation

Integral to an understanding of persister cell biology is not only the question how those bacteria enter a dormant, drug-tolerant state, but also how they can leave it again in a controlled manner. This ability distinguishes actual persister cell formation from laboratory models creating "persister-like" cells by transiently inhibiting cellular processes with bacteriostatic drugs or via the ectopic expression of toxic proteins. Though it is clear that at least some *E. coli* persisters can wake up in response to fresh nutrients, not much has been published about the seemingly stochastic resuscitation of persister cells under unchanged conditions (Allison et al., 2011, Joers et al., 2010; see also Chap. 10). Intimately connected to persister resuscitation is the repair of possible damage caused in the cell by antibiotic drugs during the period of dormancy. The role of cellular repair pathways in antibiotic tolerance has only emerged rather recently, and it is becoming clear that a simple view of persisters as hibernating cells that remain spotless upon antibiotic treatment is not true.

Cellular repair during drug tolerance has been best studied for fluoroquinolone antibiotics that cause DNA double-strand breaks by poisoning DNA processing. Not unexpectedly, fluoroquinolone tolerance of *E. coli* generally requires a functional SOS response including various SOS-controlled DNA repair functions, and pre-activating the SOS response can increase fluoroquinolone tolerance (Dörr et al., 2009, Theodore et al., 2013; see also Fig. 3.2, Goneau et al., 2014). The need for efficient DNA repair is clearly apparent from the facts that each poisoned topoisomerase complex can cause one double-strand break but that *E. coli* K-12 can only repair up to four such breaks per cell (see the article of Theodore et al., 2013, and literature cited therein). It is therefore critical that DNA lesions and poisoned topoisomerase complexes are removed from the chromosome before bacterial resuscitation (Völzing and Brynildsen,

2015). Consistently, fluoroquinolone-tolerant persisters forming during exponential growth seem to experience only modest DNA damage compared to their dying peers (Dörr et al., 2009). Only under these conditions of growing populations and with high doses of fluoroquinolone treatment could a key role of the TisB/*istR* TA module be detected, probably because it shuts down cell division and DNA processing so that the number of active replication forks is reduced and the time available for DNA repair is extended (Dörr et al., 2010, Theodore et al., 2013). Stationary phase cells are inherently non-growing and, consistently, do not show a defect in persister formation or survival without TisB/*istR* (Dörr et al., 2010). Unlike during exponential growth, the DNA damage (measured as SOS induction) caused by fluoroquinolones in stationary phase persisters does not differ significantly from the rest of the population, but the persisters have a more abundant or proficient DNA repair machinery that can repair the damage before resuscitation (Völzing and Brynildsen, 2015).

The repair of damage caused by other classes of antibiotics has been less well studied. A study performed in *Vibrio cholerae* described the WigKR two-component regulatory system as a key factor in the repair of cell wall damage caused by β-lactam antibiotics (Dörr et al., 2016). WigKR positively controls various genes involved in cell wall synthesis and remodeling, it is activated by antibiotic-induced cell wall damage, and mutants lacking this system had massively decreased levels of survivors of β-lactam treatment (Dörr et al., 2016). No comparable findings have been published for *E. coli*, but it was reported that the DpiBA two-component regulatory system would sense damage caused by β-lactam and consequently activate SOS signaling to inhibit cell division and induce drug tolerance (Miller et al., 2004). Analogous mechanisms of repair functions enabling persister survival have not been established for aminoglycoside antibiotics that poison ribosomal translation, and no obvious link to damage repair was found in a comprehensive transposon screen on aminoglycoside tolerance in *E. coli* (Shan et al., 2015). A recent study implicated trans-translation – a repair pathway rescuing stalled ribosomes – in the survival of aminoglycoside-tolerant persisters (Li et al., 2013). However, the knockout mutants deficient in trans-translation displayed lower MICs to aminoglycoside antibiotics and were also generally more sensitive to various stresses and also other classes of antibiotics, so that it is not clear whether there is a direct and specific link between cellular damage caused by aminoglycosides and trans-translation.

3.6 Discussion

This chapter summarized the classical views of persister cell biology by highlighting how *E. coli* populations use multiple molecular mechanisms to form a heterogeneous subset of persisters with a diverse profile of stress tolerance. A key conclusion is that some of these pathways are quite well understood on the molecular level but that a number of important questions remain unanswered.

For example, it is open for debate whether all the different molecular mechanisms are truly distinct or whether there is something like a "grand unified theory" of persister formation that links many or all of them. One obvious candidate for such an explanation would the drop in cellular ATP levels that different laboratories have observed for *E. coli* persisters formed by widely different mechanisms and that is driving persister formation also in *S. aureus* (Wilmaerts et al., 2018, Shan et al., 2017, Conlon et al., 2016, Dörr et al., 2010). This link would be intuitive because the processes poisoned by bactericidal antibiotics generally depend on ATP as an energy source, but a drop in cellular ATP levels among various types of persisters remains to be shown. Other researchers in the field are more skeptical about highlighting the role of specific molecular phenomena. Instead, they lean more or less strongly towards the idea that a significant proportion of persisters might just form through "PASH" (Goormaghtigh et al., 2018b, Kaldalu et al., 2016), though this view is not mutually exclusive with ATP depletion or other downstream phenomena as the actual cause of antibiotic tolerance. I believe that the continuous coexistence of these and many other very distinct views on antibiotic tolerance is primarily caused by the fragmented nature of the literature that makes it easy to cherry-pick studies supporting any imaginable viewpoint. Consequently, it would be useful to see future studies adopting some kind of standardized procedures regarding assay setups and culture media or the framework of classification and data quantification (Brauner et al., 2016, Balaban et al., 2013, Goormaghtigh et al., 2018b, Harms et al., 2017, Goormaghtigh and Van Melderen, 2016).

In addition, any interpretation or conclusion of the persister phenomenon must continuously be probed against relevant controls and alternative hypotheses. A recent study provided an interesting null hypothesis for interpreting antibiotic tolerance by proposing that treatment failure might not be linked to biological phenomena like persisters but ra-

ther be due to population heterogeneity in the interaction of antibiotics and their targets (Abel Zur Wiesch et al., 2015). In this view, the biphasic appearance of time-kill curves could be explained without invoking biologically distinct bacterial subpopulations merely by cell-to-cell variability in drug susceptibility, i.e., phenotypic resistance alone. Examples would be population heterogeneity in drug target molecules and drug uptake or action that have all been previously described (see Sect. 3.2.2).

In order to resolve these debates, future work could focus on studying the physiological parameters of genetically susceptible bacteria that survive antibiotic treatment on the single cell level. Though technically challenging, such a single-cell view would enable the direct evaluation of the relative contributions of different routes to antibiotic tolerance or phenotypic resistance. Furthermore, I feel that it is important to rely less on test tube experiments and to make particular efforts to explore and possibly prove the role of persister cells in clinical infections *in vivo*.

3.7 Acknowledgements

The author is grateful to Prof. Kenn Gerdes, Prof. Urs Jenal, Dr. Szabolcs Semsey, and Dr. Pablo Manfredi for stimulating discussions about the elusive nature of genetically encoded antibiotic tolerance. This work was supported by Swiss National Science Foundation (SNSF) Ambizione Fellowship PZ00P3_180085.

3.8 References

ABEL ZUR WIESCH, P., ABEL, S., GKOTZIS, S., OCAMPO, P., ENGELSTADTER, J., HINKLEY, T., MAGNUS, C., WALDOR, M. K., UDEKWU, K. & COHEN, T. 2015. Classic reaction kinetics can explain complex patterns of antibiotic action. *Sci Transl Med,* 7**,** 287ra73.

ALDRED, K. J., KERNS, R. J. & OSHEROFF, N. 2014. Mechanism of quinolone action and resistance. *Biochemistry,* 53**,** 1565-74.

ALLISON, K. R., BRYNILDSEN, M. P. & COLLINS, J. J. 2011. Metabolite-enabled eradication of bacterial persisters by aminoglycosides. *Nature,* 473**,** 216-20.

AMATO, S. M. & BRYNILDSEN, M. P. 2015. Persister Heterogeneity Arising from a Single Metabolic Stress. *Curr Biol,* 25**,** 2090-8.

AMATO, S. M., ORMAN, M. A. & BRYNILDSEN, M. P. 2013. Metabolic control of persister formation in *Escherichia coli*. *Mol Cell,* 50**,** 475-87.

BAHAROGLU, Z. & MAZEL, D. 2014. SOS, the formidable strategy of bacteria against aggressions. *FEMS Microbiol Rev,* 38**,** 1126-45.

BALABAN, N. Q., GERDES, K., LEWIS, K. & MCKINNEY, J. D. 2013. A problem of persistence: still more questions than answers? *Nat Rev Microbiol,* 11**,** 587-91.

BALABAN, N. Q., MERRIN, J., CHAIT, R., KOWALIK, L. & LEIBLER, S. 2004. Bacterial persistence as a phenotypic switch. *Science,* 305**,** 1622-5.

BERGHOFF, B. A., HOEKZEMA, M., AULBACH, L. & WAGNER, E. G. 2017. Two regulatory RNA elements affect TisB-dependent depolarization and persister formation. *Mol Microbiol,* 103**,** 1020-1033.

BERGHOFF, B. A. & WAGNER, E. G. H. 2017. RNA-based regulation in type I toxin-antitoxin systems and its implication for bacterial persistence. *Curr Genet,* 63**,** 1011-1016.

BIGGER, J. 1944. TREATMENT OF STAPHYLOCOCCAL INFECTIONS WITH PENICILLIN BY INTERMITTENT STERILISATION. *The Lancet,* 244**,** 497-500.

BLANGO, M. G. & MULVEY, M. A. 2010. Persistence of uropathogenic *Escherichia coli* in the face of multiple antibiotics. *Antimicrob Agents Chemother,* 54**,** 1855-63.

BRAUNER, A., FRIDMAN, O., GEFEN, O. & BALABAN, N. Q. 2016. Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nat Rev Microbiol,* 14**,** 320-30.

CHO, H., UEHARA, T. & BERNHARDT, T. G. 2014. Beta-lactam antibiotics induce a lethal malfunctioning of the bacterial cell wall synthesis machinery. *Cell,* 159**,** 1300-11.

CLAUDI, B., SPRÖTE, P., CHIRKOVA, A., PERSONNIC, N., ZANKL, J., SCHÜRMANN, N., SCHMIDT, A. & BUMANN, D. 2014. Phenotypic variation of *Salmonella* in host tissues delays eradication by antimicrobial chemotherapy. *Cell,* 158**,** 722-33.

CONLON, B. P., ROWE, S. E., GANDT, A. B., NUXOLL, A. S., DONEGAN, N. P., ZALIS, E. A., CLAIR, G., ADKINS, J. N., CHEUNG, A. L. & LEWIS, K. 2016. Persister formation in *Staphylococcus aureus* is associated with ATP depletion. *Nat Microbiol,* 1**,** 16051.

CORONA, F. & MARTINEZ, J. L. 2013. Phenotypic Resistance to Antibiotics. *Antibiotics (Basel),* 2**,** 237-55.

DÖRR, T., ALVAREZ, L., DELGADO, F., DAVIS, B. M., CAVA, F. & WALDOR, M. K. 2016. A cell wall damage response mediated by a sensor kinase/response regulator pair enables beta-lactam tolerance. *Proc Natl Acad Sci U S A,* 113**,** 404-9.

DÖRR, T., LEWIS, K. & VULIĆ, M. 2009. SOS Response Induces Persistence to Fluoroquinolones in *Escherichia coli*. *PLoS Genet,* 5**,** e1000760.

DÖRR, T., VULIC, M. & LEWIS, K. 2010. Ciprofloxacin causes persister formation by inducing the TisB toxin in *Escherichia coli*. *PLoS Biol,* 8**,** e1000317.

EL MEOUCHE, I., SIU, Y. & DUNLOP, M. J. 2016. Stochastic expression of a multiple antibiotic resistance activator confers transient resistance in single cells. *Sci Rep,* 6**,** 19538.

FAUVART, M., DE GROOTE, V. N. & MICHIELS, J. 2011. Role of persister cells in chronic infections: clinical relevance and perspectives on anti-persister therapies. *J Med Microbiol,* 60**,** 699-709.

FISHER, R. A., GOLLAN, B. & HELAINE, S. 2017. Persistent bacterial infections and persister cells. *Nat Rev Microbiol,* 15**,** 453-464.

FRIDMAN, O., GOLDBERG, A., RONIN, I., SHORESH, N. & BALABAN, N. Q. 2014. Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. *Nature,* 513**,** 418-21.

GOHARA, D. W. & YAP, M. F. 2018. Survival of the drowsiest: the hibernating 100S ribosome in bacterial stress management. *Curr Genet,* 64**,** 753-760.

GONEAU, L. W., YEOH, N. S., MACDONALD, K. W., CADIEUX, P. A., BURTON, J. P., RAZVI, H. & REID, G. 2014. Selective target inactivation rather than global metabolic dormancy causes antibiotic tolerance in uropathogens. *Antimicrob Agents Chemother,* 58**,** 2089-97.

GOORMAGHTIGH, F., FRAIKIN, N., PUTRINS, M., HALLAERT, T., HAURYLIUK, V., GARCIA-PINO, A., SJODIN, A., KASVANDIK, S., UDEKWU, K., TENSON, T., KALDALU, N. & VAN MELDEREN, L. 2018a. Reassessing the Role of Type II Toxin-Antitoxin Systems in Formation of *Escherichia coli* Type II Persister Cells. *MBio,* 9.

GOORMAGHTIGH, F., FRAIKIN, N., PUTRINS, M., HAURYLIUK, V., GARCIA-PINO, A., UDEKWU, K., TENSON, T., KALDALU, N. & VAN MELDEREN, L. 2018b. Reply to Holden and Errington, "Type II Toxin-Antitoxin Systems and Persister Cells". *MBio,* 9.

GOORMAGHTIGH, F. & VAN MELDEREN, L. 2016. Optimized Method for Measuring Persistence in *Escherichia coli* with Improved Reproducibility. *Methods Mol Biol,* 1333**,** 43-52.

GUTIERREZ, A., JAIN, S., BHARGAVA, P., HAMBLIN, M., LOBRITZ, M. A. & COLLINS, J. J. 2017. Understanding and Sensitizing Density-Dependent Persistence to Quinolone Antibiotics. *Mol Cell,* 68**,** 1147-1154 e3.

HARMS, A., BRODERSEN, D. E., MITARAI, N. & GERDES, K. 2018. Toxins, Targets, and Triggers: An Overview of Toxin-Antitoxin Biology. *Mol Cell,* 70**,** 768-784.

HARMS, A., FINO, C., SØRENSEN, M. A., SEMSEY, S. & GERDES, K. 2017. Prophages and Growth Dynamics Confound Experimental Results with Antibiotic-Tolerant Persister Cells. *MBio,* 8.

HARMS, A., MAISONNEUVE, E. & GERDES, K. 2016. Mechanisms of bacterial persistence during stress and antibiotic exposure. *Science,* 354.

HAURYLIUK, V., ATKINSON, G. C., MURAKAMI, K. S., TENSON, T. & GERDES, K. 2015. Recent functional insights into the role of (p)ppGpp in bacterial physiology. *Nat Rev Microbiol,* 13**,** 298-309.

HELAINE, S., CHEVERTON, A. M., WATSON, K. G., FAURE, L. M., MATTHEWS, S. A. & HOLDEN, D. W. 2014. Internalization of

Salmonella by macrophages induces formation of nonreplicating persisters. *Science,* 343**,** 204-8.

HOFSTEENGE, N., VAN NIMWEGEN, E. & SILANDER, O. K. 2013. Quantitative analysis of persister fractions suggests different mechanisms of formation among environmental isolates of *E. coli*. *BMC Microbiol,* 13**,** 25.

JOERS, A., KALDALU, N. & TENSON, T. 2010. The frequency of persisters in *Escherichia coli* reflects the kinetics of awakening from dormancy. *J Bacteriol,* 192**,** 3379-84.

JOHNSON, P. J. & LEVIN, B. R. 2013. Pharmacodynamics, population dynamics, and the evolution of persistence in *Staphylococcus aureus*. *PLoS Genet,* 9**,** e1003123.

KALDALU, N., HAURYLIUK, V. & TENSON, T. 2016. Persisters-as elusive as ever. *Appl Microbiol Biotechnol,* 100**,** 6545-53.

KEREN, I., KALDALU, N., SPOERING, A., WANG, Y. & LEWIS, K. 2004a. Persister cells and tolerance to antimicrobials. *FEMS Microbiol Lett,* 230**,** 13-8.

KEREN, I., SHAH, D., SPOERING, A., KALDALU, N. & LEWIS, K. 2004b. Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. *J Bacteriol,* 186**,** 8172-80.

KEREN, I., WU, Y., INOCENCIO, J., MULCAHY, L. R. & LEWIS, K. 2013. Killing by bactericidal antibiotics does not depend on reactive oxygen species. *Science,* 339**,** 1213-6.

KOHANSKI, M. A., DWYER, D. J. & COLLINS, J. J. 2010. How antibiotics kill bacteria: from targets to networks. *Nat Rev Microbiol,* 8**,** 423-35.

KOTTE, O., VOLKMER, B., RADZIKOWSKI, J. L. & HEINEMANN, M. 2014. Phenotypic bistability in *Escherichia coli*'s central carbon metabolism. *Mol Syst Biol,* 10**,** 736.

KRAUSE, K. M., SERIO, A. W., KANE, T. R. & CONNOLLY, L. E. 2016. Aminoglycosides: An Overview. *Cold Spring Harb Perspect Med,* 6.

LAXMINARAYAN, R., DUSE, A., WATTAL, C., ZAIDI, A. K., WERTHEIM, H. F., SUMPRADIT, N., VLIEGHE, E., HARA, G. L., GOULD, I. M., GOOSSENS, H., GREKO, C., SO, A. D., BIGDELI, M., TOMSON, G., WOODHOUSE, W., OMBAKA, E., PERALTA, A. Q., QAMAR, F. N., MIR, F., KARIUKI, S., BHUTTA, Z. A., COATES, A., BERGSTROM, R., WRIGHT, G. D., BROWN, E. D. & CARS, O. 2013. Antibiotic resistance-the need for global solutions. *Lancet Infect Dis,* 13**,** 1057-98.

LEE, A. J., WANG, S., MEREDITH, H. R., ZHUANG, B., DAI, Z. & YOU, L. 2018. Robust, linear correlations between growth rates and beta-lactam-mediated lysis rates. *Proc Natl Acad Sci U S A,* 115**,** 4069-4074.

LEVIN, B. R., CONCEPCION-ACEVEDO, J. & UDEKWU, K. I. 2014. Persistence: a copacetic and parsimonious hypothesis for the existence of non-inherited resistance to antibiotics. *Curr Opin Microbiol,* 21**,** 18-21.

LEVIN, B. R. & ROZEN, D. E. 2006. Non-inherited antibiotic resistance. *Nat Rev Microbiol,* 4**,** 556-62.

LEWIS, K. 2005. Persister cells and the riddle of biofilm survival. *Biochemistry (Mosc),* 70**,** 267-74.

LEWIS, K. 2010. Persister cells. *Annu Rev Microbiol,* 64**,** 357-72.

LI, J., JI, L., SHI, W., XIE, J. & ZHANG, Y. 2013. Trans-translation mediates tolerance to multiple antibiotics and stresses in *Escherichia coli*. *J Antimicrob Chemother,* 68**,** 2477-81.

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**,** 2092-9.

LIU, Y. & IMLAY, J. A. 2013. Cell death from antibiotics without the involvement of reactive oxygen species. *Science,* 339**,** 1210-3.

LUIDALEPP, H., JOERS, 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**,** 3598-605.

MA, C., SIM, S., SHI, W., DU, L., XING, D. & ZHANG, Y. 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.

MAISONNEUVE, E. & GERDES, K. 2014. Molecular mechanisms underlying bacterial persisters. *Cell,* 157**,** 539-48.

MCKAY, S. L. & PORTNOY, D. A. 2015. Ribosome hibernation facilitates tolerance of stationary-phase bacteria to aminoglycosides. *Antimicrob Agents Chemother,* 59**,** 6992-9.

MICHIELS, J. E., VAN DEN BERGH, B., VERSTRAETEN, N., FAUVART, M. & MICHIELS, J. 2016. *In vitro* emergence of high persistence upon periodic aminoglycoside challenge in the ESKAPE pathogens. *Antimicrob Agents Chemother,* 60**,** 4630-7.

MILLER, C., THOMSEN, L. E., GAGGERO, C., MOSSERI, R., INGMER, H. & COHEN, S. N. 2004. SOS response induction by ß-lactams and bacterial defense against antibiotic lethality. *Science,* 305**,** 1629-31.

MOLINA-QUIROZ, R. C., LAZINSKI, D. W., CAMILLI, A. & LEVY, S. B. 2016. Transposon-Sequencing Analysis Unveils Novel Genes Involved in the Generation of Persister Cells in Uropathogenic *Escherichia coli*. *Antimicrob Agents Chemother,* 60**,** 6907-6910.

MORDUKHOVA, E. A. & PAN, J. G. 2014. Stabilization of homoserine-O-succinyltransferase (MetA) decreases the frequency of persisters in *Escherichia coli* under stressful conditions. *PLoS One,* 9**,** e110504.

MOYED, H. S. & BERTRAND, K. P. 1983. *hipA*, a newly recognized gene of *Escherichia coli* K-12 that affects frequency of persistence after inhibition of murein synthesis. *J Bacteriol,* 155**,** 768-75.

NEIDHARDT, F. C. 2006. Apples, oranges and unknown fruit. *Nat Rev Microbiol,* 4**,** 876.

NGUYEN, D., JOSHI-DATAR, A., LEPINE, F., BAUERLE, E., OLAKANMI, O., BEER, K., MCKAY, G., SIEHNEL, R., SCHAFHAUSER, J., WANG, Y., BRITIGAN, B. E. & SINGH, P. K. 2011. Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. *Science,* 334**,** 982-6.

OCAMPO, P. S., LAZAR, V., PAPP, B., ARNOLDINI, M., ABEL ZUR WIESCH, P., BUSA-FEKETE, R., FEKETE, G., PAL, C., ACKERMANN, M. & BONHOEFFER, S. 2014. Antagonism between bacteriostatic and bactericidal antibiotics is prevalent. *Antimicrob Agents Chemother,* 58**,** 4573-82.

ORMAN, M. A. & BRYNILDSEN, M. P. 2013. Dormancy is not necessary or sufficient for bacterial persistence. *Antimicrob Agents Chemother,* 57**,** 3230-9.

PENNINGTON, J. M. & ROSENBERG, S. M. 2007. Spontaneous DNA breakage in single living *Escherichia coli* cells. *Nat Genet,* 39**,** 797-802.

PU, Y., ZHAO, Z., LI, Y., ZOU, J., MA, Q., ZHAO, Y., KE, Y., ZHU, Y., CHEN, H., BAKER, M. A., GE, H., SUN, Y., XIE, X. S. & BAI, F. 2016. Enhanced Efflux Activity Facilitates Drug Tolerance in Dormant Bacterial Cells. *Mol Cell,* 62**,** 284-94.

RENGGLI, S., KECK, W., JENAL, U. & RITZ, D. 2013. Role of autofluorescence in flow cytometric analysis of *Escherichia coli* treated with bactericidal antibiotics. *J Bacteriol,* 195**,** 4067-73.

SCHUMACHER, M. A., BALANI, P., MIN, J., CHINNAM, N. B., HANSEN, S., VULIC, M., LEWIS, K. & BRENNAN, R. G. 2015. HipBA-promoter structures reveal the basis of heritable multidrug tolerance. *Nature,* 524**,** 59-64.

SHAN, Y., BROWN GANDT, A., ROWE, S. E., DEISINGER, J. P., CONLON, B. P. & LEWIS, K. 2017. ATP-Dependent Persister Formation in *Escherichia coli*. *MBio,* 8.

SHAN, Y., LAZINSKI, D., ROWE, S., CAMILLI, A. & LEWIS, K. 2015. Genetic basis of persister tolerance to aminoglycosides in *Escherichia coli*. *MBio,* 6.

SPOERING, A. L., VULIC, M. & LEWIS, K. 2006. GlpD and PlsB participate in persister cell formation in *Escherichia coli*. *J Bacteriol,* 188**,** 5136-44.

STEPANYAN, K., WENSELEERS, T., DUENEZ-GUZMAN, E. A., MURATORI, F., VAN DEN BERGH, B., VERSTRAETEN, N., DE MEESTER, L., VERSTREPEN, K. J., FAUVART, M. & MICHIELS, J. 2015. Fitness trade-offs explain low levels of persister cells in the opportunistic pathogen *Pseudomonas aeruginosa*. *Mol Ecol,* 24**,** 1572-83.

STEWART, B. & ROZEN, D. E. 2012. Genetic variation for antibiotic persistence in *Escherichia coli*. *Evolution,* 66**,** 933-9.

THEODORE, A., LEWIS, K. & VULIC, M. 2013. Tolerance of *Escherichia coli* to fluoroquinolone antibiotics depends on specific components of the SOS response pathway. *Genetics,* 195**,** 1265-76.

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.

VAN DEN BERGH, B., MICHIELS, J. E., WENSELEERS, T., WINDELS, E. M., BOER, P. V., KESTEMONT, D., DE MEESTER, L., VERSTREPEN, K. J., VERSTRAETEN, N., FAUVART, M. & MICHIELS, J. 2016. Frequency of antibiotic application drives rapid evolutionary adaptation of *Escherichia coli* persistence. *Nat Microbiol,* 1**,** 16020.

VAN MELDEREN, L. & WOOD, T. K. 2017. Commentary: What Is the Link between Stringent Response, Endoribonuclease Encoding Type II Toxin-Antitoxin Systems and Persistence? *Front Microbiol,* 8.

VAZQUEZ-LASLOP, N., LEE, H. & NEYFAKH, A. A. 2006. Increased persistence in *Escherichia coli* caused by controlled expression of toxins or other unrelated proteins. *J Bacteriol,* 188**,** 3494-7.

VEENING, J. W., SMITS, W. K. & KUIPERS, O. P. 2008. Bistability, epigenetics, and bet-hedging in bacteria. *Annu Rev Microbiol,* 62**,** 193-210.

VEGA, N. M., ALLISON, K. R., KHALIL, A. S. & COLLINS, J. J. 2012. Signaling-mediated bacterial persister formation. *Nat Chem Biol,* 8**,** 431-3.

VERSTRAETEN, N., KNAPEN, W. J., KINT, C. I., LIEBENS, V., VAN DEN BERGH, B., DEWACHTER, L., MICHIELS, J. E., FU, Q., DAVID, C. C., FIERRO, A. C., MARCHAL, K., BEIRLANT, J., VERSEES, W., HOFKENS, J., JANSEN, M., FAUVART, M. & MICHIELS, J. 2015. Obg and Membrane Depolarization Are Part of a Microbial Bet-Hedging Strategy that Leads to Antibiotic Tolerance. *Mol Cell,* 59**,** 9-21.

VÖLZING, K. G. & BRYNILDSEN, M. P. 2015. Stationary-Phase Persisters to Ofloxacin Sustain DNA Damage and Require Repair Systems Only during Recovery. *MBio,* 6**,** e00731-15.

WAKAMOTO, Y., DHAR, N., CHAIT, R., SCHNEIDER, K., SIGNORINO-GELO, F., LEIBLER, S. & MCKINNEY, J. D. 2013. Dynamic persistence of antibiotic-stressed mycobacteria. *Science,* 339**,** 91-5.

WILMAERTS, D., BAYOUMI, M., DEWACHTER, L., KNAPEN, W., MIKA, J. T., HOFKENS, J., DEDECKER, P., MAGLIA, G., VERSTRAETEN, N. & MICHIELS, J. 2018. The Persistence-Inducing Toxin HokB Forms Dynamic Pores That Cause ATP Leakage. *MBio,* 9.

WIUFF, C. & ANDERSSON, D. I. 2007. Antibiotic treatment in vitro of phenotypically tolerant bacterial populations. *J Antimicrob Chemother,* 59**,** 254-63.

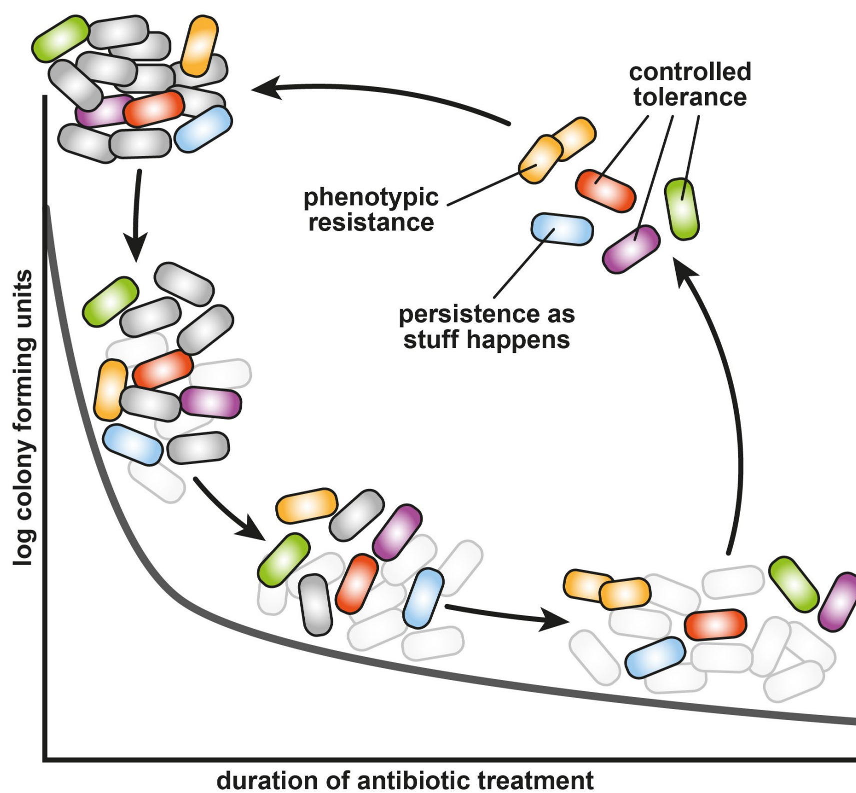
WOOD, T. K., KNABEL, S. J. & KWAN, B. W. 2013. Bacterial persister cell formation and dormancy. *Appl Environ Microbiol,* 79**,** 7116-21.

YANG, J. H., BENING, S. C. & COLLINS, J. J. 2017. Antibiotic efficacy-context matters. *Curr Opin Microbiol,* 39**,** 73-80.

3.9 Figure Captions

Fig 3.1. Heterogeneity of cells surviving antibiotic treatment.

The illustration shows the typical biphasic appearance of a time kill curve with the rapid death of most regularly growing cells (grey, turning colorless) followed by a second phase of slower killing. Surviving cells can have a wide variety of physiological properties (colors). Drug-tolerant, dormant persisters can arise either in a controlled manner via different molecular pathways (green / red / violet) or through cellular accidents («persistence as stuff happens»; blue). Phenotypically resistant bacteria are not dormant and can divide in presence of the antibiotic (yellow). After treatment, surviving persisters can resuscitate and replenish the population.



Author's accepted manuscript

Fig 3.2. Multiple molecular mechanisms underlying the survival of antibiotic treatment.

The illustration highlights how starvation or stress can induce the formation of antibiotic-tolerant cells (colored) among a population of susceptible peers (grey) through responsive diversification. Different molecular mechanisms enabling the survival of antibiotic treatment are highlighted schematically. PG = peptidoglycan, PMF = proton-motive force