AMERICAN SOCIETY FOR MICROBIOLOGY | Microbiology and Molecular Biology Reviews®

REVIEW

In Vitro Studies of Persister Cells

Niilo Kaldalu,[a] Vasili Hauryliuk,[a,b,c] Kathryn Jane Turnbull,[b,c] Agnese La Mensa,[a] Marta Putriņš,[a] Tanel Tenson[a]

[a]Institute of Technology, University of Tartu, Tartu, Estonia
[b]Department of Molecular Biology, Umeå University, Umeå, Sweden
[c]Laboratory for Molecular Infection Medicine Sweden, Umeå University, Umeå, Sweden

SUMMARY Many bacterial pathogens can permanently colonize their host and establish either chronic or recurrent infections that the immune system and antimicrobial therapies fail to eradicate. Antibiotic persisters (persister cells) are believed to be among the factors that make these infections challenging. Persisters are subpopulations of bacteria which survive treatment with bactericidal antibiotics in otherwise antibiotic-sensitive cultures and were extensively studied in a hope to discover the mechanisms that cause treatment failures in chronically infected patients; however, most of these studies were conducted in the test tube. Research into antibiotic persistence has uncovered large intrapopulation heterogeneity of bacterial growth and regrowth but has not identified essential, dedicated molecular mechanisms of antibiotic persistence. Diverse factors and stresses that inhibit bacterial growth reduce killing of the bulk population and may also increase the persister subpopulation, implying that an array of mechanisms are present. Hopefully, further studies under conditions that simulate the key aspects of persistent infections will lead to identifying target mechanisms for effective therapeutic solutions.

KEYWORDS antibiotic resistance, antimicrobial agents, persistence

INTRODUCTION

The term "persisters" (persister cells) is used to refer to individual bacteria that survive antibiotic treatment, which otherwise kills the large majority of their kin population (1, 2). Treatment with bactericidal antibiotics very rarely kills 100% of the bacterial cells, and persisters are common in different microbial populations and communities. Strictly speaking, this term designates the minor subpopulation of bac-

Citation Kaldalu N, Hauryliuk V, Turnbull KJ, La Mensa A, Putriņš M, Tenson T. 2020. *In vitro* studies of persister cells. Microbiol Mol Biol Rev 84:e00070-20. https://doi.org/10.1128/MMBR.00070-20.

Copyright © 2020 American Society for Microbiology. All Rights Reserved.

Address correspondence to Niilo Kaldalu, niilo.kaldalu@ut.ee.

Published 11 November 2020

Downloaded from https://journals.asm.org/journal/mmbr on 07 June 2021 by 193.40.12.11.

teria that are transiently tolerant to the lethal activity of antimicrobials in a test tube. In the host, bacteria (both persisters and nonpersisters) can evade effective treatment by multiple factors relating to patient and drug (3). Here, we provide an overview of persister research to those readers who find that papers on the topic can be confusing and contradictory. We emphasize essential research that is consensually accepted, while communicating also the conflicting data.

Many fundamental observations and ideas about persisters originate from very early papers and are still generally valid. Therefore, we built the story around a few seminal papers and focus on two interwoven topics: (i) the different growth behavior of individual bacteria in an isogenic population and (ii) the molecular mechanisms of persistence.

DEFINING ANTIBIOTIC RESISTANCE, TOLERANCE, AND PERSISTENCE

To understand persistence, it is crucial to distinguish the two modes of antibiotic action. Both bacteriostatic and bactericidal antibiotics stop growth of sensitive bacterial populations as a direct result of binding to the target (4). However, bactericidal antibiotics kill bacteria by inducing toxic malfunctioning (corruption [5]) of the targeted process (6, 7). The bacterial ability to resist growth inhibition is referred to as "antibiotic resistance," while the ability of a bacterial population to survive the duration of an antibiotic treatment is dubbed "antibiotic tolerance" (see the glossary in the Appendix). Importantly, while antibiotic tolerance describes slow killing (reduced killing rate) of the bulk bacterial population (1), "persistence" and "heteroresistance" refer to a small fraction of the population that is either tolerant (persisters) or resistant to antibiotics, respectively (8).

Antibiotic tolerance inversely correlates with bacterial growth rate (9–12) and can be mechanistically explained by low activity of the target and/or downstream processes which diminish the corruption by the antibiotic. Antibiotic tolerance is either genotypic or phenotypic (12). Genotypic tolerance characterizes bacterial isolates or mutants that, under similar conditions, are killed at a reduced rate in comparison to the standard- or wild-type strains (12, 13). Just like resistant mutants are selected during antibacterial therapy, antibiotic treatment can select for the mutants that show increased tolerance to killing by the drug (13). Often, these mutants display a growth defect compared to the parental strain. Phenotypic tolerance occurs in response to an environmental trigger, primarily in circumstances of slow growth and in nongrowing conditions. For example, the nongrowing bacteria of a starved culture survive treatment with β-lactams and glycopeptides, which are able to effectively kill the isogenic strain during normal growth conditions (14). These groups of antibiotics inhibit the cell wall synthesis—a process that is required for growth but is relatively inactive in the nongrowing cells (Table 1).

Antibiotic resistance and tolerance levels often vary between individual cells or subpopulations and those of the bulk population. Bacteria exhibit phenotypic heterogeneity: even in the same environment, they can be phenotypically diverse and show differences in gene expression, shape, and metabolism (15–18). Differences in growth rate of individual bacteria in a clonal population have a strong effect on antibiotic susceptibility (19–23). An extreme example of such heterogeneity is the formation of endospores in sporulating bacterial species. These nonreplicating survival forms endure harsh environmental stresses, including antibiotics (24). In a similar way, populations of nonsporulating microbes contain slowly growing and nongrowing cells that show increased tolerance to antibiotics.

ESSENTIALS OF PERSISTER CELLS

Persisters are an extremely antibiotic-tolerant subpopulation of bacterial cells. Many single-cell observations have demonstrated that these cells are in a transiently nongrowing state (19–21, 25). While the nongrowing state of persisters explains their extreme tolerance to antibiotics, the growth arrest alone does not explain their tolerance to agents that act on pathways unrelated to growth, such as fluoroquinolones

Downloaded from https://journals.asm.org/journal/mmbr on 07 June 2021 by 193.40.12.11.

TABLE 1 The targets and modes of action of the main classes of bactericidal antibiotics

Bactericidal antibiotics	Targets and modes of action
β-Lactams	Disrupt cell wall synthesis: penicillins, cephalosporins, monobactams, and carbapenems. Bind covalently to the enzymes that synthesize and reorganize the bacterial cell wall: peptidoglycan synthetases and hydrolases (penicillin binding proteins [PBPs]). Induce bacterial lysis and abnormal cell shape (234).
Quinolones	Clinically important drugs are fluoroquinolones, e.g., ciprofloxacin, levofloxacin, ofloxacin, and gatifloxacin. Bind to the complexes of type II topoisomerases and DNA at the stage where both cleaved DNA strands are covalently attached to the topoisomerase subunits. Induce DNA brakes, SOS response, and mutagenesis in targeted bacteria (230).
Aminoglycosides	Target protein synthesis, bind to the decoding center of the small (30S) ribosomal subunit. Bactericidal action is caused by erroneous translation, particularly mistranslation of membrane protein leading to membrane damage (6, 231, 232).
Peptide antibiotics	Nonribosomally synthesized peptides: glycopeptides (e.g., vancomycin), polymyxins (e.g., colistin), gramicidins, and bacitracins. Target cell wall synthesis and cell membranes; clinical use is restricted due to their toxicity and a narrow spectrum of action (233).
Other classes	Diverse mechanisms of action: macrolides (e.g., erythromycin), chloramphenicol, and rifampin are bactericidal against some bacterial phyla/strains and bacteriostatic against others. The antituberculosis (pro)drug isoniazid is bactericidal against fast-growing and bacteriostatic against slow-growing mycobacteria. When activated by KatG, it inhibits synthesis of mycolic acids and the mycobacterial cell wall. An antituberculosis drug, bedaquiline of the diarylquinoline class, blocks the mycobacterial ATP synthesis.

and aminoglycosides (26). Usually—but not always—persisters have entered a nongrowing state already before the antibiotic treatment starts (1). It is important to note that persisters are not resistant, as they cannot proliferate in the presence of the antibiotic (Fig. 1 and 2; see the glossary in the Appendix). Persistence is not heritable; persister cells are phenotypic bacterial variants that are genetically identical to the sensitive bacteria. Therefore, their progeny is sensitive to killing by antibiotics and contains a small fraction of persisters as all other bacterial populations do (27, 28).

Persistent forms of bacterial pathogens are believed to be important contributors toward the failure of antimicrobial therapy of prolonged and recurrent infections (3), such as biofilm formation *in vivo* (29, 30), tuberculosis (31), urinary tract infections (32), and lung infections in cystic fibrosis patients (33). Antibiotic tolerance also facilitates the evolution and spread of resistance (13, 34–38). Despite that, the actual significance of persisters in an infection has remained elusive: their role in the causation and recalcitrance of diseases is hard to estimate. Therefore, in this review, we leave infections aside and focus on *in vitro* studies that were conducted to identify the molecular mechanisms of persistence. For this purpose, persisters have been studied extensively in *Escherichia coli* K-12 laboratory strains. This model organism enabled simple cultivation, genetic manipulation, and use of the following single-cell techniques: fluorescent reporters, microfluidics, and live imaging microscopy (19, 20, 39–41). Ambig-

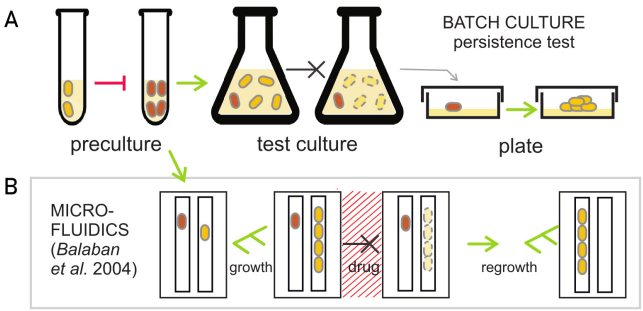

FIG 1 The nongrowing state of persisters was discovered using time-lapse microscopy. (A and B) The patterns of bacterial fate that occur in typical batch cultures during growth, antibiotic treatment, and evaluation of survival by plating (A) were observed under the microscope by tracking division and lysis of individual bacteria (19) (B). *E. coli* was grown in a microfluidics device, treated with a β-lactam antibiotic, and thereafter, allowed to regrow in the drug-free medium. The growing, antibiotic-sensitive bacteria are yellow; the nongrowing bacteria are brown. The red repression arrow marks the stress signal (starvation) that triggers the growth arrest, the green arrows mark resuscitation, and the black crosses mark killing by antibiotics.

Downloaded from https://journals.asm.org/journal/mmbr on 07 June 2021 by 193.40.12.11.

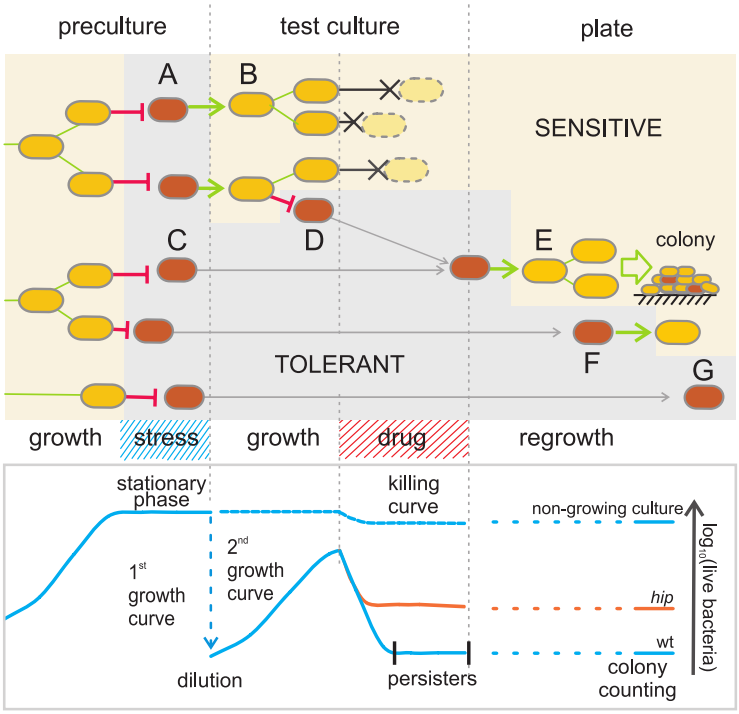

FIG 2 Persisters are transiently growth-arrested and regrow after antibiotic treatment. Upon depletion of nutrients, the (pre)culture enters the stationary phase. Bacteria stop growing (red bar; A) and acquire antibiotic tolerance (gray background). Upon inoculation into fresh growth medium, the bacteria resuscitate (green arrow; B), become sensitive to antibiotics (yellow background), and are killed during bactericidal treatment (black cross). A fraction of the inoculated bacteria (triggered persisters) maintain the nongrowing state they acquired during starvation in the preculture, despite the fresh supply of nutrients (C). Spontaneous persisters become growth-arrested during exponential growth (D). Persisters survive antibacterial treatment and resuscitate afterward (E), forming colonies. A fraction of the nongrowing cells maintain membrane integrity and metabolic activity but resuscitate too late to form visible colonies (F) or stay growth-arrested (G) (23, 25). The bottom panel shows the growth and killing curves of the antibiotic-treated cultures. In growing cultures, killing of proliferating bacteria forms the slope of the bi-phasic killing curve, and persisters form the plateau stage, which with *hip* mutants, is considerably higher than in the wild type (101). The nongrowing (stationary-phase) cultures and biofilms survive bactericidal drug treatments much better than the growing cultures (90).

uous and often contradictory results of these studies imply that multiple processes contribute to persister formation and resuscitation. Some researchers hypothesize that persistence occurs as an inadvertent result of various errors and glitches, which happen randomly, temporarily obstructing proliferation of some bacteria (42, 43). Others continue a quest to search for dedicated mechanisms that underlie the observable ambiguity (44–47) and might inspire better antibacterial therapy. In reality, it is likely that both scenarios contribute toward persister cell formation.

THE DISCOVERY OF PERSISTERS

The pioneering studies of penicillin (β-lactam; Table 1) showed the antibiotic to be bactericidal (48, 49). In 1944, Joseph Bigger found that a small fraction of bacteria survived and regrew after the penicillin treatment of cultured staphylococci (28). He called these surviving cells "persisters" and demonstrated that, unlike resistant mutants, they produce drug-sensitive progeny.

Bigger has put forward two important hypotheses.

First, he observed that penicillin was only partially effective against staphylococcal osteomyelitis, and the disease recurred after the secession of treatment. Drug-sensitive, causative bacteria were subsequently isolated from the treated patients. Bigger then suggested that persistence is not merely a laboratory artifact, but a reason for antimicrobial treatment failure. Therefore, if clinically relevant, persisters in patients may be similar to those that occur in a test tube, and understanding the mechanisms of

Downloaded from https://journals.asm.org/journal/mmbr on 07 June 2021 by 193.40.12.11.

persistence in culture would lead to effective therapy of hard-to-treat infections. This hypothesis has driven persister studies since that time.

Second, Bigger noticed that growth-inhibited and slow-growing cultures are cleared inefficiently. He hypothesized that penicillin kills only dividing bacteria and that persister cells are in a nonreplicating phase. Technical limitations at this time meant that he had no means to observe the proliferation and killing of individual bacteria, and the nonproliferation of persisters was only a hypothesis (1). Numerous researchers went on to support this hypothesis experimentally using advanced techniques, and nonproliferation became recognized as a fundamental characteristic of persister cells (19–21, 25, 39, 50).

THE TRANSIENT NONGROWING STATE OF PERSISTERS

Nathalie Balaban and her colleagues from the Stanislas Leibler lab made persisters visible and established that they are truly nonproliferating (19). In their work, they used *E. coli* high-persistence (*hip*) mutants (we will discuss these mutants further later in the text), grew them in a microfluidic device, and tracked individual cells using time-lapse microscopy. A small number of nongrowing cells were observed among the growing *hipA7* mutant culture. Upon treatment with ampicillin (β-lactam; Table 1), the replicating cells were lysed, while the few nongrowers were not. After the removal of ampicillin, some (but not all) of these nongrowing cells started to proliferate in the drug-free medium and were identified as persisters.

The *hipA7* persisters detected in this experiment were growth-arrested starting from their inoculation into the microfluidics chamber. Their nongrowing state was retained from the stationary-phase seed culture and was not drug-induced (Fig. 1). The authors categorized such "preexisting" persistence as type I (19), which was recently changed to "triggered persistence," emphasizing that it is induced by stress, e.g., starvation in the stationary phase (1). In other words, Balaban and colleagues demonstrated that individual bacteria have different durations of the lag phase. Persisters "get stuck" in the nongrowing state, survive the antibiotic treatment, and later spontaneously switch to the growth phenotype (Fig. 1). Heterogeneous exit of individual bacteria from the lag phase happens also on agar plates and can be monitored using an automated colony appearance assay (51, 52). Flow cytometry enabled tracking of both the dividing and the nongrowing bacteria in liquid cultures, using dilution of presynthesized fluorescent protein (21, 25). This method demonstrated that growing cultures contain large fractions of nongrowing cells that are alive but unable to regrow (Fig. 2). The number of these cells can exceed the number of colony-forming persisters by orders of magnitude (53, 54), illustrating that persisters cannot be equated with nongrowing cells, which may have maintained their membrane integrity and metabolic activity but irreversibly lost their capacity to resume growth when plated.

PERSISTENCE AND HETERORESISTANCE

In the same seminal microfluidics study, Balaban and colleagues (19) also used another *E. coli hip* mutant: the *hipQ* strain. The strain is poorly characterized; the mutated genes and molecular mechanism conferring the phenotype are unknown (55). In this strain, the surviving cells were not completely growth-arrested but grew and divided continuously at a rate about 10 times lower than the sensitive cells, even at a high antibiotic concentration. According to the present terminology, the *hipQ* strain might be called heteroresistant (see the glossary in the Appendix). However, it must be noted that bacterial elongation in the presence of the antibiotic is not enough to prove resistance. Cells often elongate in response to drugs such as fluoroquinolones and subsequently die. Thus, at this point, it is still unclear if the *hipQ* subpopulation survive because of their slowed growth or unknown resistance factors.

Heteroresistance is caused by heterogeneous expression of resistance factors and is often related to transient amplification of parts of the bacterial chromosome (56). Similar to persistence, it is widespread in different bacterial phyla and may potentially cause treatment failure, though its clinical relevance has been debated (57). For

Downloaded from https://journals.asm.org/journal/mmbr on 07 June 2021 by 193.40.12.11.

example, vancomycin heteroresistance in *Staphylococcus aureus* has spread among hospitalized patients and can develop into hereditary vancomycin resistance upon exposure to the drug (58). According to one study, it was associated with a treatment failure in a patient with endocarditis and in the rabbit model of endocarditis (59), while another study found no effect of heteroresistance on the outcome of the treatment of bacteremia (60). Beyond *S. aureus*, heteroresistance to the last-line polymyxin antibiotic colistin was identified in a clinical isolate of the nosocomial pathogen *Enterobacter cloacae* (61). While colistin effectively rescued the mice infected with a susceptible strain, those infected with the heteroresistant isolates failed the therapy and died (62). Studies of heteroresistance suggest that "stochastic expression of any factor that facilitates or opposes the action of an antibiotic could influence the fate of single cells" (63). For example, microfluidic cultures of *Mycobacterium smegmatis* were shown to maintain a stable number of living cells in in the presence of isoniazid, a drug that is activated by the catalase-peroxidase KatG. The apparent stability was a result of balanced division and death due to stochastic expression of KatG in different cells (63). Furthermore, phenotypically resistant isolates of *Salmonella enterica* are able to survive lethal concentrations of nalidixic acid due to heterogeneous efflux pump activity and survive kanamycin treatment due to the heterogeneity in expression of the porin OmpC, which is required for permeation of the drug through the outer membrane (64). Phenotypic resistance against antibiotics with low membrane permeability can be caused by biochemical memory effects that establish bistable bacterial growth rates (65). In summary, heteroresistance is another type of noninherited heterogeneity that can potentially save bacterial populations from eradication (8, 57).

STRESS-TRIGGERED AND SPONTANEOUS PERSISTENCE

Conditions that cause inhibition of growth induce tolerance of the bulk of the bacterial population to several groups of antibiotics (e.g., β-lactams [14]) and, in some cases, are also associated with an increase in persister frequency. Common persistence-associated factors are intracellular infection (reviewed in reference 3) and starvation. Importantly, bacteria present in overnight seed cultures are starved before they are transferred into fresh, nutrient-rich medium. In fresh medium, these cells need time to adapt, and therefore start growing with a delay, termed the lag phase. As previously discussed, individual cells exhibit different lag times, and some live cells may have lost the ability to regrow irreversibly. The longer a seed culture is kept under nutrient limitation (in the stationary phase), the higher the incidence of persister cells in the test culture (54). When a growing culture is approaching the stationary phase and becomes nutrient limited, the number of persisters increases rapidly again (27). It is therefore essential to standardize the growth phase, growth rate, and seed culture parameters when studying persistence (39, 54, 66, 67).

Besides starvation, the bactericidal efficacy of antibiotics can be diminished by other growth-inhibiting stresses, e.g., nutritional/diauxic shift (68–72), bacteriostatic antibiotics (43, 73–77), and low cytoplasmic Mg^{2+} concentration (43). Low concentrations of bactericidal antibiotics, in particular, fluoroquinolones and aminoglycosides, also trigger persistence (78–82). Some studies report an effect of the pH (21, 83, 84) and osmotic shock (85, 86) on bacterial survival after antibiotic exposure, but these effects may vary among the bacterial species and conditions tested (43, 47). However, many of these studies do not distinguish antibiotic tolerance caused by arrested growth of the whole population from persistence that is caused by the absence of growth in a subpopulation. Making such a distinction would require time-kill curves that display the slope corresponding to the dying bulk and the plateau of persisters instead of the endpoint measurements of survival.

Persisters form spontaneously during steady-state exponential growth through a switch of dividing cells into a growth-arrested state; however, spontaneous persisters seem to be less common than triggered persisters (1). Repeated cycles of dilution and growth result in a drastic decrease of the persister fraction in *E. coli*, indicating that few persisters are generated during logarithmic growth (27). Still, some cells that stopped

Downloaded from https://journals.asm.org/journal/mmbr on 07 June 2021 by 193.40.12.11.

proliferation and became persisters were directly observed when a microfluidic device was seeded with exponentially growing *E. coli* (20, 39). In *M. smegmatis*, repeated passaging does not eliminate persisters, suggesting that the frequency of spontaneous persisters may vary among bacterial species, and they might form a majority of persisters in this organism (87). In conclusion, we can say that multiple environmental triggers induce temporary growth arrest in a fraction of bacteria, and a fraction of these nongrowing microbes, in turn, become persisters. In addition, persisters may form spontaneously.

DORMANCY AND PHYSIOLOGICAL ACTIVITY OF PERSISTERS

It is generally accepted that persisters survive bactericidal treatments due to dormancy (2). However, defining "dormancy" is not trivial. This term is commonly used to refer to the nongrowing or slow-growing state but also implies a lack of or slow metabolic activity. However, multiple studies indicate that persisters maintain a level of macromolecule synthesis and retain limited metabolic activity. These processes include active protein synthesis, sugar metabolism, and DNA winding by topoisomerases (26, 88, 89). Furthermore, persisters also require ATP-dependent protein disaggregation when exiting the persister state (45). Importantly, these processes can be considered potential targets in combatting persistent infections.

In certain media, aminoglycosides (AG; Table 1) kill 100% of *P. aeruginosa* and *E. coli* cells, including persister cells that survive treatments by other bactericidal antibiotics (23, 89, 90). Eradication of persisters by AG in glucose-containing media shows that *E. coli* persisters take up and metabolize glucose, which in turn, creates a membrane potential of sufficient magnitude to enable the transport of positively charged AG into bacterial cells. In media containing alternate carbon sources, e.g., arabinose, persisters survive AG treatment, demonstrating that these carbon sources are either not metabolized or do not permeate persister cells (89). As AG directly target the translation machinery, killing by AG can be seen as an indication of active protein synthesis in persister cells. Alternatively, protein synthesis might still be completely inhibited in the persister state, and the bacteria may die after the treatment when resuming growth, because of the AG that was taken up earlier during the treatment and is not pumped out fast enough during recovery. Notably, AG effectively kill only persisters in growing cultures; their bactericidal activity against starving (e.g., stationary-phase) cells and biofilms is weak (90).

Killing of nonreplicating bacteria by fluoroquinolones (FQ; Table 1) demonstrates that topoisomerases are active and vulnerable in these cells. Some FQ (e.g., ofloxacin and gatifloxacin) kill a large fraction of nongrowing stationary-phase bacteria, while others (e.g., norfloxacin) kill only growing bacteria. Certain FQ (tosufloxacin, clinafloxacin, and sparfloxacin) can eradicate persisters that survive ofloxacin treatment (91, 92). The causes of the different killing efficiencies are unknown. A study of ofloxacin-treated stationary-phase *E. coli* revealed that surviving cells (stationary-phase persisters) suffered antibiotic-induced damage, and their DNA repair machinery (SOS response) was activated upon regrowth (26, 88). When the FQ treatment was followed by nutrient deprivation, nearly 100% of the bacteria survived, showing that the timing of SOS response and resuscitation is critical for the recovery of persisters (88). As a part of the SOS response, FQ induce filamentation of the rod-shaped bacteria. While the dying cells filament during the treatment, the FQ-treated persisters retained the normal shape until the end of the treatment and formed long polynucleoid filaments upon resuscitation, which indicates the DNA damage suffered in persister stage (20).

Active protein disaggregation has a role in resuscitation of dormant bacteria. Endogenous aggregates of misfolded proteins form in many nonreplicating bacteria during the stationary phase (93). Upon regrowth, these protein aggregates are cleared with the assistance of ATP-dependent DnaK-ClpB chaperones (45, 86).

Finally, active drug efflux has been stated to contribute to persister survival. A decreased intracellular concentration of antibiotic and enhanced drug efflux in *E. coli* persisters has been reported (46) but has yet to undergo independent substantiation.

Downloaded from https://journals.asm.org/journal/mmbr on 07 June 2021 by 193.40.12.11.

If this observation were to stand scrutiny, the nonreproductive state and efflux together would define persisters; otherwise, the increased drug efflux would enable proliferation and cause resistance.

It must be noted that several studies demonstrate *in vivo* persisters to be metabolically active in infection models. Measurements of the single-cell dynamics of *Mycobacterium tuberculosis* replication and expression of rRNA revealed heterogeneity, which was amplified by nutrient limitation, intracellular replication, and growth in mouse lungs. The lungs of chronically infected mice were shown to harbor a subpopulation of nongrowing but metabolically active bacteria, which became prominent in mice treated with the antituberculotic drug isoniazid, suggesting a role in posttherapeutic relapses (94). The intracellular persisters of *Salmonella* were proven to be nongrowing but maintain a metabolically active state and reprogram the macrophages by means of secreted effectors that inhibit proinflammatory innate immune responses and induce anti-inflammatory macrophage polarization (95). The intracellular *Staphylococcus aureus* persisters within infected macrophages were shown to be in a nondividing state when followed at the single-cell level. At the same time, they remained metabolically active and displayed reduced but active translation demonstrated by inducible green fluorescent protein (GFP) production (96).

THE SEARCH FOR MECHANISMS OF PERSISTENCE LEADS TO *hip* MUTANTS

Once the growth-arrested nature of the persister state was demonstrated, investigations into the underlying mechanisms that render these rare cells nonmultiplying were invigorated. Genes required for complex phenotypes, e.g., sporulation, are usually discovered through selection of the loss-of-function mutants (97), but no mutants incapable of persister formation had been identified by screening of bacterial knockout strain libraries (98–100). Thus, researchers tried to uncover persistence mechanisms by studying mutants that show heightened persistence. Almost 40 years after Bigger's discovery, Harris Moyed and Kevin Bertrand isolated the first *hip* mutants (the aforementioned high-persistence mutants) of *E. coli* K-12 (101). Their aim was to find mutants with no increase in MIC (see the glossary in the Appendix) that would survive antibiotic treatment better than the wild type and would not be growth impaired. An increased MIC would indicate heritable resistance, and growth impairment would predictably increase antibiotic tolerance of the bulk. Using chemical mutagenesis and several passages with ampicillin (β-lactam; Table 1), followed by regrowth on drug-free agar plates, they selected for a mutated variant of the previously uncharacterized *hipA* gene that increased the persister fraction 10^3- to 10^4-fold. The use of solid media in this study allowed for counting of the live bacteria (Fig. 1). The resultant killing curves had a biphasic shape; the initial rapid killing of the sensitive bacteria was followed by a slow decline in CFU numbers (the "plateau" of persisters). While the *hip* mutant and wild-type strains exhibited equal killing rates of the sensitive population, the plateau of the *hip* mutant was about a thousandfold higher (Fig. 2).

The ampicillin-selected *hip* mutants also presented increased persistence to DNA-targeting treatments (e.g., quinolone antibiotics; Table 1) (102). Similarly, the quinolone-selected *hip* mutants had elevated persistence against β-lactams (55). This led to the conclusion that persisters are multidrug tolerant. In contrast to these first observations, numerous subsequent studies demonstrated that persister levels depend considerably on the class of the antibiotic used and that different bacterial subpopulations can be tolerant to different drugs, suggesting that persisters are not physiologically homogenous (68, 81, 103).

THE *hip* ALLELE IS A MUTANT TOXIN-ANTITOXIN SYSTEM

The *hip* mutant *hipA7* was much studied in a hope to uncover a universal persistence mechanism. The *hip* locus was shown to consist of two protein-encoding genes—*hipA* and *hipB*—which form a bicistronic operon. Moyed and colleagues found that the HipA protein is toxic to the *hipB*-deficient strains and forms a tight complex with the HipB protein (104, 105). In fact, they described the first toxin-antitoxin (TA)

Downloaded from https://journals.asm.org/journal/mmbr on 07 June 2021 by 193.40.12.11.

system (reviewed in the following section) on a bacterial chromosome, although they did not use this term at the time. Later, the growth-inhibiting HipA protein was demonstrated to be a serine/threonine kinase that phosphorylates the glutamyl-tRNA-synthetase GltX. This leads to inhibition of tRNA aminoacylation and protein synthesis (106, 107). Resolved structures of higher-order HipA-HipB-promoter assembly revealed that HipA-HipA dimerization blocks the active sites of the protein in DNA-bound complexes and is the mechanism of inhibition of its toxicity (108). The high-persistence allele *hipA7* (101) encodes a mutant form of HipA that phosphorylates the same target (109). The mutated amino acids weaken HipA dimerization on DNA and thereby unleash it to facilitate persistence (108). As for several other persistence phenotypes, the effect of *hipA7* is dependent on the age of the inoculum; when the starter culture has spent 18 h or more in the stationary phase, the mutant and wild-type strains display equal persistence (54). Mutations identical to the *in vitro*-selected *hipA7* and an additional high-persistence allele of *hipA* have been identified in *E. coli* urinary tract infection (UTI)-related and commensal strains. Deletion of the *hipA7* allele in a UTI isolate caused a decline in bacterial survival after antibiotic treatment both in bacterial cultures growing in broth and in infected culture of human bladder cells (108). Although these data suggest that *hipA* mutations could play a role in treatment difficulties of UTIs, to our knowledge, there are currently no studies looking for a correlation between the *hipA* mutations and UTI treatment outcome.

Eventually, the studies of the *hip* mutant did not identify genes or pathways that are essential for persister formation. Deletion of *hipBA* did not affect persister frequency in either growing or stationary-phase cultures (5, 54). The initially reported decrease of persistence of stationary-phase cultures and biofilms of a Δ*hipBA* strain (5) was due to extension of the deletion into the nearby *dif* (chromosome partitioning) region (100). As far as we know, there is currently no example of a *hip*-like mechanism—increase in persister fraction but no increase in the antibiotic tolerance of bulk—that is proven to be responsible for recalcitrance of a persistent infection. Cumulative evidence suggests that unrelated mutations in any bacterial gene, which result in slow growth or delay in poststress resuscitation, decrease bactericidal efficacy of antibiotics. These mutants are consequently under positive selection when survival of lethal stress outweighs fast growth (13).

THE ROLE OF TOXIN-ANTITOXIN MODULES IN PERSISTENCE

After mapping the first *hip* mutations to the *hipBA* locus, TA systems became favored candidates for mechanistic persistence effectors. TA systems consist of toxins that stop growth by targeting vital bacterial functions and are coexpressed with antitoxins that preclude their activity (110). In type II TA systems, which resemble HipBA, antitoxins are proteins that bind to and inhibit cognate toxins and simultaneously control transcription of the TA operon.

Previously, TA systems were characterized as plasmid stabilization systems (111), but the functions of the many newly discovered chromosomal TA systems were puzzling, and their deletions had no known phenotype (112). One hypothesis suggested that toxins of TA system are active only in persister cells and are directly responsible for their transitory growth arrest. Theoretically, the self-regulated TA systems look like ideal candidates for persistence switches. The TA mRNA induction in nonreplicating bacterial subpopulations that were isolated through induced lysis of sensitive bacteria (5, 113) or cell sorting (114) was interpreted as a potential indicator of the toxin activation in persister cells (5), while recent findings show that an increase of TA transcript levels does not necessarily indicate liberation of the toxin (115).

While individual deletions of TA operons had no effect on persistence, the multiple deletion of 10 type II TA systems in *E. coli* was reported to reduce it significantly, although it did not eliminate persisters completely (116). Further, TA-dependent persistence was attributed to a stochastic increase of (p)ppGpp concentration in a few cells, which was, in turn, proposed to induce the proteolysis of antitoxins (117). This model was accepted as a major mechanism of persistence (118, 119) and stimulated

Downloaded from https://journals.asm.org/journal/mmbr on 07 June 2021 by 193.40.12.11.

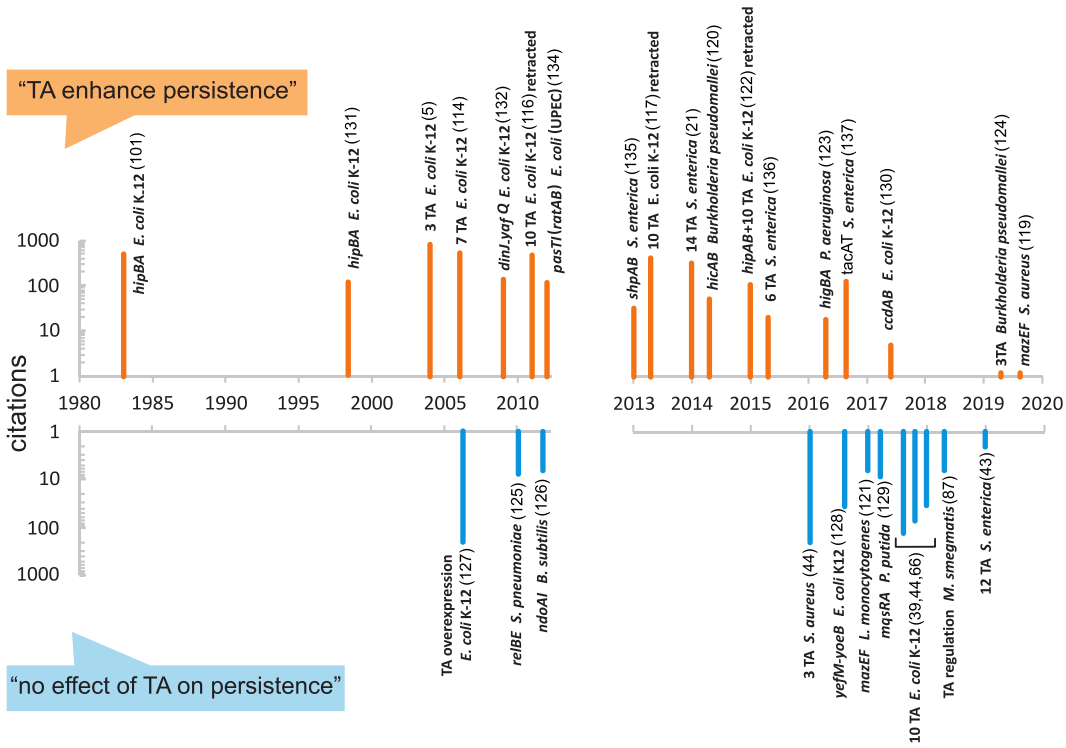

FIG 3 Testing the role of TA in persistence. Experimental studies that have suggested (5, 101, 114, 131) or reported the positive effect of TA on persistence are marked by upward bars (orange) in the order of their publication. Downward bars (blue) mark studies that did not see an effect of TA on persister formation and/or critically reassessed the published evidence or interpretation. The length of the bar corresponds to the number of citations (in log scale) of each publication on Google Scholar as of November 2019.

more research on TA systems. TA-mediated persistence was reported in different bacterial species, although some researchers did not see the anticipated effects (Fig. 3) (120–136). Some studies even found that certain TA systems enhance the lethal effect of antibiotics in *Streptococcus pneumoniae* and *Bacillus subtilis* (125, 126). Bacteria that were growth-arrested by artificially overproduced toxins were often used as "model persisters" to circumvent the challenging work on a tiny subpopulation. That was done despite the demonstration that overexpression of other, unrelated, proteins may induce growth arrest and antibiotic tolerance as well (127).

Ultimately, the model of TA-mediated persistence in *E. coli* did not stand up to scrutiny (39, 66). The effects were not reproducible in more carefully constructed multiple-deletion strains and were explained by bacteriophage infection, activation of prophages, and technical irregularities in the experiments (66). The apparent induction of TA modules and (p)ppGpp in persister cells was a result of using inadequate fluorescent reporters (39). The studies of effects of TA systems on persistence and antibiotic tolerance of *Salmonella*, an organism that is very similar to *E. coli*, have given diverse results. A study reported a decrease of macrophage-induced nonreplicating bacteria and persisters in 12 different single TA deletion mutants (21), while an independent study that was performed in a test tube did not detect effects of these individual TA deletions but found a severalfold reduction in survival of the Δ12TA multiple deletion mutant (43). One of the toxins of *Salmonella*, TacT, works by acetylating aminoacyl tRNAs, whereas hydrolysis of corrupted tRNA by peptidyl-tRNA hydrolase Pth was reported to resuscitate persisters (137). Another study that suggests a role of TA specifically upon macrophage infection was carried out in *Burkholderia pseudomallei*. No effect for single TA deletions was detected in test tube-grown bacterial cultures, but decreased survival of the antibiotic treatment occurred in macrophages (124). While the reasons for the different experimental outcomes are

Downloaded from https://journals.asm.org/journal/mmbr on 07 June 2021 by 193.40.12.11.

often unclear, such discrepancies should encourage researchers to perform complementation tests of TA gene knockouts and reassess the effects of the type II TA system on persistence. The inconsistent results of persister assays are often caused by variation of the experimental conditions, and precise trial protocols have helped to overcome these issues (51, 52, 66, 67, 138).

Another class of TA system, type I, has also been linked to persistence. Toxins of these TA systems are, typically, small proteins which target cell membranes, causing membrane depolarization and ATP depletion. Translation of these toxins is suppressed by antisense sRNA antitoxins (139). An example of a type I TA system that promotes persistence is the TisB/IstR-1 system of *E. coli*. This TA system is integrated into the SOS regulon, is induced by fluoroquinolone antibiotics (FQ; Table 1), and enhances persistence to FQ (78, 79, 140). The promoter of the *tisB* toxin gene is repressed by LexA, the master regulator of the SOS response and is induced upon DNA damage (79). This leads to the formation of two subpopulations of bacteria, polarized and depolarized cells (140). Deletion of *tisB* decreases persistence to FQ (79), while deletion of the regulatory RNA sequences produces a highly persistent (*hip*) strain (140). In response to overproduction of the GTPase Obg in *E. coli*, a TisB-like toxin, HokB (of the *hokB-sokB* type I TA system), was shown to form pores and enhance survival of antibiotic treatment (141), while the reverse process, HokB monomerization and membrane repolarization, leads to reversal of this phenotype (142). The relevance of TisB/IstR-1-like systems to persistence of bacterial pathogens during infection is unknown.

(p)ppGpp SIGNALING AND PERSISTENCE

The signal nucleotide (p)ppGpp has been proposed to function as a master regulator of persister formation (119, 143). (p)ppGpp reprograms bacterial physiology in response to nutrient limitation and is overproduced in the stationary phase (144). It binds to and controls multiple cellular targets (145–147). Increased (p)ppGpp levels promote catabolic reactions (148) and repress growth (149, 150) by inhibiting protein synthesis and ribosome assembly (151), as well as transcription of rRNA and ribosomal proteins (152). Transcriptional inhibition occurs directly, by binding of (p)ppGpp to RNA polymerase (153, 154), and indirectly, by depleting the GTP pool (144). In the stationary phase, increased (p)ppGpp levels induce formation of inactive 100S ribosome dimers (155, 156). (p)ppGpp-mediated signaling is also important in the formation of antibiotic-tolerant biofilms in *E. coli* (157), *Enterococcus* (158), and *Vibrio cholerae* (159).

A key question is how to separate the direct effects of (p)ppGpp from the inevitable effects of stresses that induce production of this regulator. The connection between antibiotic tolerance and (p)ppGpp signaling emerged with the discovery of *rel* (relaxed) mutants (160). The property of penicillin to kill only actively dividing bacteria (49) was used in *E. coli* genetics for isolating auxotrophic mutants (161, 162) and *rel* regulatory mutants (160). These experiments identified the *relA* gene that codes for an enzyme synthesizing guanosine(penta)tetraphosphate [(p)ppGpp] (147).

The intracellular levels of (p)ppGpp are controlled by RelA/SpoT homolog (RSH) enzymes (163). Experiments in (p)ppGpp null mutants have shown that persisters can also form in the absence of the regulatory nucleotide (70, 143), but given how central (p)ppGpp is for control of bacterial metabolism, its effects are extremely pleiotropic (164, 165). While the knockouts commonly cause a significant decrease in the levels of persistence (68, 70, 143, 166), in some organisms (and under certain conditions) deletion of the RSH genes had no effect (43, 44) or even enhanced antibiotic tolerance (167). (p)ppGpp deficiency thoroughly disturbs bacterial physiology, particularly upon stress and in biofilms. A complete absence of (p)ppGpp causes multiple amino acid requirements, poor survival of aged cultures, and aberrant cell morphology and motility, as well as defects of membrane permeability and a delay in growth rate reduction response upon entry into starvation (43, 150, 168). In *P. aeruginosa*, a lack of (p)ppGpp severely reduced persistence of stationary-phase cultures and biofilms but caused spontaneous cell death and autolysis of starving cells under the same conditions (143). In *E. coli*, disruption of the *relA* gene delays regrowth when bacteria are provided with

Downloaded from https://journals.asm.org/journal/mmbr on 07 June 2021 by 193.40.12.11.

fresh nutrients after starvation and, therefore, causes a transient *increase* in tolerance to β-lactams in a medium-specific manner (167), the same phenomenon was used to isolate the early *rel* mutants (160). The reports implicating (p)ppGpp-mediated signaling in stochastic persister formation via activation of TA systems (117, 122) in *E. coli* were later reassessed (67, 118) and eventually retracted. However, this does not reject the possibility of (p)ppGpp- and TA-mediated persistence or antibiotic tolerance as such. As we mentioned above, the HipA toxin phosphorylates the glutamyl-tRNA-synthetase GltX that leads to a shortage of aminoacylated tRNA and activates (p)ppGpp synthesis by RelA (stringent response) (106, 107). The (p)ppGpp induction and *relA* are required to enhance the growth arrest and antibiotic tolerance by ectopically expressed *hipA* (106, 169). A new group of TA systems that are composed of *bona fide* (p)ppGpp-synthesizing RSH toxins, which are paired with neutralizing antitoxin proteins, was described recently (170), adding another layer of complexity to the puzzle of signal nucleotides and TA. In conclusion, RSH gene disruption is too "heavy-handed" an approach for establishing the specific role of (p)ppGpp in persistence, and these studies were not able to conclusively evaluate the involvement of (p)ppGpp in persister cell formation.

A more direct way to assess the role of (p)ppGpp in persister formation is to measure (p)ppGpp concentration at the single-cell level and to correlate these observations with the cell's commitment to persistence. This requires time-resolved quantification, since the (p)ppGpp concentration is capable of reaching maximum levels (and dropping back to the baseline levels) in a matter of minutes (167). Unfortunately, an adequate experimental tool for this is lacking. While the RpoS-mCherry translational fusion has been repeatedly used as a proxy for intracellular (p)ppGpp concentration (117, 122, 166), there is no strict proof that this fusion construct exclusively reports ppGpp levels. Time resolution of the fusion protein is limited by slow mCherry maturation (20 to 40 min in *E. coli* [171]) and its accumulation in stable aggregates (39, 166). Its fluorescence must be constantly tracked to locate *de novo* fluorescent cells; otherwise, it mostly marks the bacteria that retained the nondividing state since prior to the stationary phase (39). A recently discovered RNA riboswitch that specifically binds (p)ppGpp (172) could serve as a foundation for designing time-resolved (p)ppGpp reporters.

While *in vitro* investigation of (p)ppGpp's role in persistence is convoluted, the clinical evidence of selective pressure acting on the RSH genes and promoting antibiotic tolerance is well documented. Truncated versions of the Rel RSH enzyme, which constitutively produce (p)ppGpp, were shown to be responsible for antibiotic tolerance of *S. aureus* (173) and *Enterococcus faecium* (174) clinical isolates. A mutation in *rel*, resulting in reduced (p)ppGpp hydrolase activity, was identified in a β-lactam-tolerant *S. aureus* clinical strain (174). However, in all of these cases, increased (p)ppGpp levels induced the antibiotic tolerance of bulk population, and therefore, none of these mutants can be considered true *hip* mutants.

PERSISTENCE WITHOUT A DEDICATED MECHANISM?

Screening of knockout (98–100, 175) and expression (176) libraries did not identify specialized persistence pathways but showed that several genes and pathways differing in primary function affect persister level. Stochastic fluctuation in gene expression may produce bi-stable phenotypes and subpopulations that show different levels of antibiotic tolerance. For example, individual cells of *S. aureus* expressing lower levels of the tricarboxylic acid (TCA) cycle enzymes exhibited higher antibiotic tolerance (177). The newly selected *hip* mutations of *E. coli* mapped to genes of several metabolic enzymes, stress response proteins, and antitoxins, showing that persistence can be attributed to different mechanisms (34, 178). While no special persistence mechanisms have been identified, Bruce Levin and colleagues have formulated the persistence as stuff happens (PaSH) hypothesis. They postulate that persistence is an inadvertent product of different errors in cell division. The genes that contribute to it modulate the rate at which these errors occur and/or are corrected (42). The theory resembles the ideas of Thomas Nyström, who attributed bacterial growth arrest to senescence and

Downloaded from https://journals.asm.org/journal/mmbr on 07 June 2021 by 193.40.12.11.

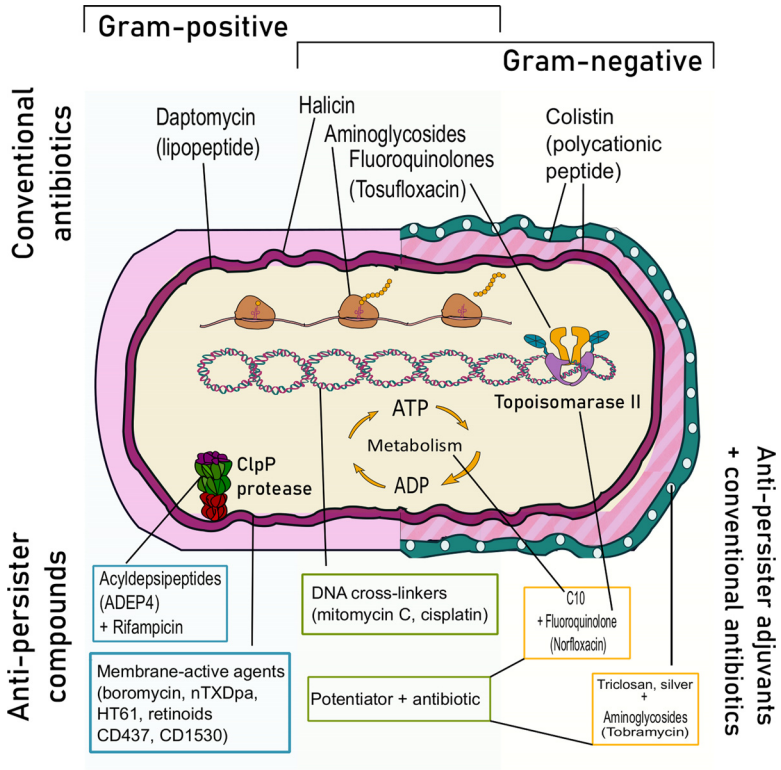

FIG 4 Antipersister compounds. Several newly discovered compounds and conventional antibiotics of different classes have antipersister activity. They kill a large fraction of persisters of both Gram-negative and Gram-positive species which have survived bactericidal treatment with common bactericidal antibiotics (e.g., ampicillin or ofloxacin). Some antipersister compounds are active as adjuvants in combination with known bactericidal antibiotics.

protein damage (179). However, the PaSH model does not answer every question. It does not explain mechanistically the apparent coordination between different cellular processes during persister formation and regrowth. Furthermore, it does not specify how errors in cell division (and repair) are communicated to the translational and metabolic machinery in a way that ultimately causes either antibiotic tolerance or leads to resuscitation of the bacterial cell.

DRUGS AND STRATEGIES AGAINST PERSISTERS

Numerous studies have been conducted to isolate antipersister agents against *in vitro*-formed persisters in a hope to make extrapolations about their clinical benefit (180, 181). The devised compounds aim to either (i) directly kill dormant persister cells or (ii) resensitize/resuscitate them to antibiotic-susceptible states. While antibiotics usually target growth-related synthetic processes (Table 1), several antipersister agents target essential components of the cell in a growth-independent fashion (Fig. 4). The antipersister and cytotoxic concentrations of the thus identified compounds are close, which severely limits their therapeutic potential, in the best case to topical applications (181). Despite that, several of them are enrolled in clinical trials (180). The anti-tumor DNA cross-linking agents mitomycin C and cisplatin have demonstrated activity against nongrowing cultures, biofilms (5), and persisters (182, 183). Antipersister activity against Gram-positive microbes and/or mycobacteria was demonstrated for different membrane-active agents—quinolone-like compounds (184), synthetic retinoids (also *in vivo*) (185), and others (186, 187). While Gram-positive bacterial membranes contain a substantial fraction of anionic lipids, mammalian membranes consist of zwitterionic neutral lipids and have high levels of cholesterol, which enables a degree of selectivity for the membrane-disrupting antimicrobial agents (188, 189). The Eleftherios Mylonakis lab screened for the membrane-targeting antimicrobials that rescued infected *Caeno-*

Downloaded from https://journals.asm.org/journal/mmbr on 07 June 2021 by 193.40.12.11.

rhabditis elegans, thus identifying the compounds that did not kill the nematodes and had a lower toxicity (185, 186).

Antipersister compounds that potentiate conventional antibiotics may stimulate metabolic activity of *in vitro* persisters, reverting them to an antimicrobial-susceptible state, as does the fatty acid signaling molecule *cis*-2-decenoic acid (190), or facilitate the uptake of antibiotic into persister cells such as triclosan (a disinfectant) and silver in the case of aminoglycosides (128, 191). C10, a small molecule that potentiates killing of Gram-negative pathogens by the FQ norfloxacin (192), inhibits *M. tuberculosis* respiration, blocks its tolerance to isoniazid (INH), and restores INH sensitivity in otherwise INH-resistant *M. tuberculosis* strains *in vitro* (193). Hopefully, at least some of these listed compounds or combinations show activity against *bona fide* persisters of medically relevant organisms within the infected host, proving their clinical relevance.

As we have mentioned, under particular conditions, some already licensed antibiotics can kill a large fraction of the nongrowing bacteria or totally eradicate them *in vitro*. The activity of conventional antibiotics and their combinations against the *in vitro*-generated persisters that survive ampicillin or ofloxacin treatment has been demonstrated in several studies. Certain fluoroquinolones (FQ), foremost tosufloxacin, are highly active against the persisters of uropathogenic *E. coli* (UPEC) (92) and *S. aureus* (91). Alas, these FQ have been withdrawn or their clinical development was stopped due to toxicity (194). *E. coli* persisters were killed by a combination of aminoglycosides and colistin, and *S. aureus* persisters were killed by aminoglycosides plus daptomycin (81). A recent study found that sequential treatment of *E. coli* by a combination of an antibiotic that is strongly dependent on metabolism (ampicillin or ciprofloxacin) and a second antibiotic that is weakly dependent on metabolism but normally not used because of toxicity (mitomycin C and colistin) can sterilize the culture, killing both metabolically active and persister cells, while simultaneously dose-sparing (195). Some new or repurposed antimicrobials were shown to kill nongrowing bacteria, including *in vitro* persisters. Halicin, originally developed as an antidiabetes agent and recently identified as a candidate broad-spectrum antibiotic that dissipates the ΔpH component of the proton motive force, is bactericidal against a wide spectrum of pathogens and eradicates *E. coli* persisters (196). Researchers from the Kim Lewis lab found that the ribosomally encoded cyclic peptide lassomycin kills *M. tuberculosis*, including the dormant mycobacteria, by targeting the ATP-dependent protease ClpC1P1P2 (197), while the antimicrobial peptide ADEP4 (acyldepsipeptide), which reprograms the ClpP protease to nonselectively degrade cellular proteins in a ATP-independent manner (198, 199), can kill methicillin-resistant *S. aureus* (MRSA) persisters and, in combination with rifampin, eradicates them completely (200). Most importantly, ADEP4 with rifampin eradicated staphylococcal infection in a deep-seated mouse thigh infection model, promising a path toward developing therapies (200).

An example of a new antibiotic that was demonstrated to be active against *M. tuberculosis* persisters is bedaquiline, which is used in combination regimens against multidrug-resistant tuberculosis (201). When combined with other antituberculosis medications, it killed persisters both *in vitro* (202) and *in vivo* (203). In ongoing clinical trials, it has led to high rates and shorter times of conversion to negative sputum culture (204, 205). Bedaquiline is a diarylquinoline compound with a novel mechanism of action that targets the ATP synthase (206). It has both bactericidal and sterilizing activities demonstrated in a mouse tuberculosis (TB) model (207, 208). Killing of *M. tuberculosis* persisters with no relapse was demonstrated for a bedaquiline-containing regimen compared to the conventional anti-TB multidrug regimen in a mouse model (203). It killed those *M. tuberculosis in vivo* persisters, which could not be detected by a regular culture (209), but resuscitated and formed colonies when stimulated by *M. tuberculosis* culture supernatant (210), which contains secreted proteins known as resuscitation-promoting factors (211). Another new anti-TB drug, delamanid, which blocks the production of mycolic acids, thus destabilizing the cell wall (212), killed nonreplicating *M. tuberculosis in vitro* and in a guinea pig model of TB (213). These results give hope for a faster cure with reduced relapse rate.

Downloaded from https://journals.asm.org/journal/mmbr on 07 June 2021 by 193.40.12.11.

CONCLUSIONS AND PERSPECTIVES

In 2001, Kim Lewis proposed the role of persisters in the antibiotic tolerance of biofilms, prompting research into this neglected topic (214). Studies of persister cells have provided much knowledge regarding the heterogeneity of bacterial growth *in vitro* but much less information about such heterogeneity upon persistent infections. The complexity of infection cannot be fully reproduced in a test tube, and we do not know whether those persisters we observe *in vitro* resemble physiologically the bacteria that cause treatment failures during persistent infections.

How has the study of antibiotic persisters contributed to elucidating the complexity of persistent infection? During infection, bacteria encounter several stresses that may induce the persister state, e.g., phagocytosis (21), and location in acidic compartments (83). Antibiotic treatment can modulate these responses, for example, by inducing formation of a nonreplicating bacterial subpopulation that is tolerant to both antibiotics and the host complement system (215). Several studies have shown how bacteria can persist during antibiotic treatment in infection models, not due to the persister cells but due to transient phenotypic resistance (22, 216–218). For example, the major contributors to the progression of *Salmonella* infection in an animal model were not growth-arrested but were slowly growing and moderately antibiotic-tolerant cells (22). According to accepted models, *M. tuberculosis* infections persist during antibiotic treatment because of nonreplicating mycobacteria (219), while a study of *Mycobacterium marinum*-infected zebrafish larvae and *M. tuberculosis*-infected cultured macrophages demonstrated survival of actively replicating subpopulations. These mycobacteria expressed macrophage-induced drug efflux pumps at a high level and maintained transient phenotypic resistance for at least 120 h after macrophage lysis (216). Another phenotypic switch that allows bacteria to avoid antibacterial action is the formation of cell wall-deficient L-forms under isotonic conditions. L-forms are resistant to cell wall-targeting antibiotics and switch back to the walled state following antibiotic withdrawal (217). L-forms have been observed in macrophages, animal models, and recently, urine of patients with recurrent urinary tract *E. coli* infections (rUTI) (218). In conclusion, the study of persisters has certainly helped develop bacterial cell biology methods that allow the investigation of complex bacterial heterogeneity that could reflect the complexities of persistent infection. Interest in antibiotic persisters has highlighted transient mechanisms that enable bacteria to evade antibiotic action and facilitate emergence of heritable antibiotic resistance.

Despite many attempts, the study of antibiotic persisters *in vitro* has not identified any specific mechanisms of antibiotic tolerance that could become new targets of antimicrobial therapy (Fig. 5). Is this a failure? The study of clinical isolates suggests that it might, to the contrary, reflect the challenging reality of chronic and recurrent infections. A unique source for understanding persistent infections are the longitudinal isolates of *P. aeruginosa* collected from tens of cystic fibrosis (CF) patients over many years. Characterization of the genomes and phenotypes of these isolates did not reveal a single major mechanism that might be responsible for persistence. Instead, varied mutations led to diversification and adaptation of the colonizing pathogen (220, 221). These data, together with metagenome, transcriptome, and proteome data (222–224), suggest that we should study the antibiotic susceptibility of persistent pathogens (e.g., biofilms, bacterial aggregates, intracellular bacteria—in different host cell compartments etc.) under conditions that simulate the key aspects of the host environment. In a recent review, leading experts in the field of antimicrobial resistance, advise testing of antibiotic susceptibility of important pathogens under various conditions that reflect the relevant body niche. That should include conditions of slow growth, nonreplication, and the lag periods following nonreplication and preceding resumption of growth (225).

The route that has been successful for the development of nonantimicrobial drugs starts from pinpointing drug targets and continues with high-throughput screening of chemical libraries to find their specific inhibitors. Such approaches have provided zero

Downloaded from https://journals.asm.org/journal/mmbr on 07 June 2021 by 193.40.12.11.

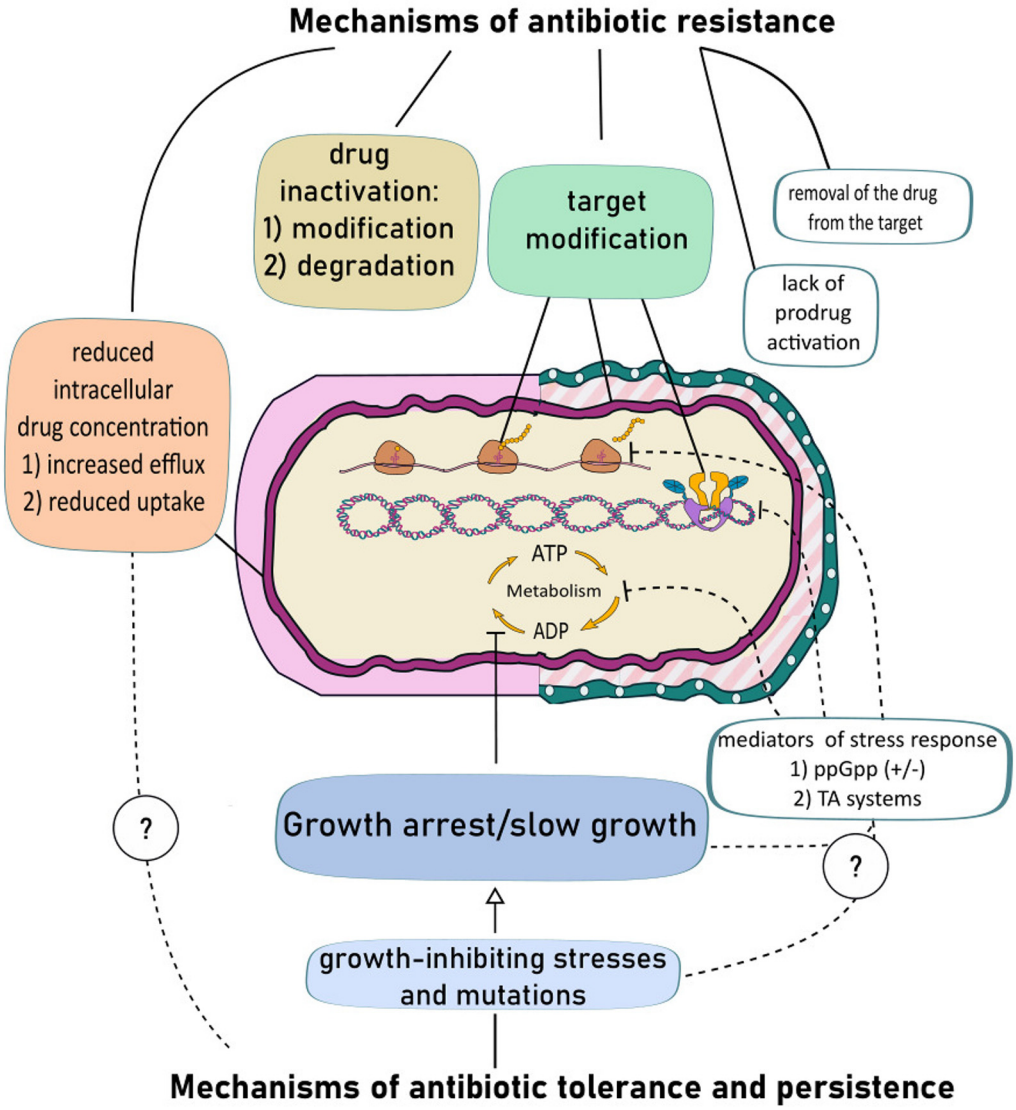

FIG 5 Growth arrest is essential for antibiotic tolerance. The molecular mechanisms of heritable antibiotic resistance are well characterized. Despite efforts, no specific molecular pathways responsible for persister formation or antibiotic tolerance of the bulk of a bacterial population have been identified. Different stresses and mutations that inhibit growth increase antibiotic tolerance and persistence. Mediators of stress response [TA systems and (p)ppGpp] inhibit the targets of antibiotics and may enhance persistence but are not essential for it.

new antibiotics despite identifying highly potent inhibitors of essential bacterial proteins. The promising leads were not effective antibacterial agents, mostly because of permeability issues and fast development of resistance (226). Thus, the outlook of target-based drug discovery against persisters and persistent infections is poor (227). Because bacteria have coexisted with other bacteria and fungi in nature for billions of years, current thinking suggests that nature has already identified the best targets and selected for the best lead antibacterial structures. Most major classes of antibiotics originate from environmental microbes and were discovered at the beginning of the antibiotic era without any knowledge of their targets or mechanisms of action. Searches for natural products and synthetic compounds that target and kill specifically the growth-arrested/nongrowing bacteria, either alone or in combination with known antimicrobials, could identify entirely unknown classes of antimicrobial substances.

APPENDIX

GLOSSARY

- **antibiotic resistance** The ability of bacteria to grow (proliferate) in the pres-

Downloaded from https://journals.asm.org/journal/mmbr on 07 June 2021 by 193.40.12.11.

ence of increased concentrations of antibiotics. The cause of the current antibiotic crisis. If not specified as "phenotypic or heteroresistance," this trait is heritable, common to all bacteria of a clonal population and relies on specific resistance genes or mutations.

- **antibiotic tolerance** The ability of bacteria to survive treatment with bactericidal antibiotics with no change in the MIC.
- ***in vitro*** In this review, *in vitro* refers to bacterial cultures in a controlled environment outside an infected animal or patient. Such use of this term is common in microbiology; in biochemistry and molecular biology, it marks procedures outside a living cell using purified components.
- ***in vivo*** In this review, *in vivo* refers to bacteria within a patient or animal model.
- **MBC** Minimum bactericidal concentration; the lowest concentration of an antibiotic required to kill ≥99.9% of the bacteria; a measure of antibiotic tolerance.
- **MDK** Minimum duration of killing; the shortest period required to kill a certain fraction (e.g., ≥99%, ≥99.9%) of the bacteria by antibiotic; a measure of antibiotic tolerance.
- **MIC** Minimum inhibitory concentration; the lowest concentration of an antibiotic required to prevent the replication of bacteria. MIC is the most common measure of antibiotic sensitivity or resistance levels. A heightened MIC value indicates resistance.
- **persistent infections** Infections caused by pathogens that remain viable in the host for a long period; either asymptomatic (subclinical) or symptomatic (3).
- **persisters (persister cells)** A subpopulation of bacteria that is highly tolerant to antibiotics and survives a treatment that kills the majority of bacteria of the clonal population.
- **phenotypic resistance** A nonheritable form of antibiotic resistance; the transient ability of otherwise susceptible bacteria to grow in the presence of antibiotics. A common form of phenotypic resistance is heteroresistance, in which a subpopulation of a clonal population shows reduced antibiotic sensitivity, frequently due to unstable genetic changes (8, 228, 229).

ACKNOWLEDGMENTS

The work was supported by the European Union from the European Regional Development Fund through the Centre of Excellence in Molecular Cell Engineering (2014-2020.4.01.15-0013), by the European Commission (EC) via project MIBEst H2020-WIDESPREAD-2018-2020/GA number 857518, and by a grant from the Estonian Research Council (PRG335) to T.T. N.K was supported by the Institute of Technology basic funding grant. V.H. was supported by the Swedish Research council via grants 2017-03783 (project grant) and 2018-00956 (within the framework of JPIAMR consortium grant RIBOTARGET).

We do not have a conflict of interest to declare.

REFERENCES

1. Balaban NQ, Helaine S, Lewis K, Ackermann M, Aldridge B, Andersson DI, Brynildsen MP, Bumann D, Camilli A, Collins JJ, Dehio C, Fortune S, Ghigo JM, Hardt WD, Harms A, Heinemann M, Hung DT, Jenal U, Levin BR, Michiels J, Storz G, Tan MW, Tenson T, Van Melderen L, Zinkernagel A. 2019. Definitions and guidelines for research on antibiotic persistence. Nat Rev Microbiol 17:441–448. https://doi.org/10.1038/s41579-019-0196-3.
2. Lewis K. 2010. Persister cells. Annu Rev Microbiol 64:357–372. https://doi.org/10.1146/annurev.micro.112408.134306.
3. Fisher RA, Gollan B, Helaine S. 2017. Persistent bacterial infections and persister cells. Nat Rev Microbiol 15:453–464. https://doi.org/10.1038/nrmicro.2017.42.
4. Walsh C, Wencewicz T. 2016. Antibiotics: challenges, mechanisms, opportunities. 2nd ed. ASM Press, Washington, DC.
5. Keren I, Shah D, Spoering A, Kaldalu N, Lewis K. 2004. Specialized persister cells and the mechanism of multidrug tolerance in Escherichia coli. J Bacteriol 186:8172–8180. https://doi.org/10.1128/JB.186.24.8172-8180.2004.
6. Davis BD, Chen LL, Tai PC. 1986. Misread protein creates membrane channels: an essential step in the bactericidal action of aminoglycosides. Proc Natl Acad Sci U S A 83:6164–6168. https://doi.org/10.1073/pnas.83.16.6164.
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. https://doi.org/10.1016/j.cell.2014.11.017.
8. Dewachter L, Fauvart M, Michiels J. 2019. Bacterial heterogeneity and antibiotic survival: understanding and combatting persistence and het-

Downloaded from https://journals.asm.org/journal/mmbr on 07 June 2021 by 193.40.12.11.

eroresistance. Mol Cell 76:255–267. https://doi.org/10.1016/j.molcel.2019.09.028.
9. Eng RHK, Padberg FT, Smith SM, Tan EN, Cherubin CE. 1991. Bactericidal effects of antibiotics on slowly growing and nongrowing bacteria. Antimicrob Agents Chemother 35:1824–1828. https://doi.org/10.1128/aac.35.9.1824.
10. Levin BR, Rozen DE. 2006. Non-inherited antibiotic resistance. Nat Rev Microbiol 4:556–562. https://doi.org/10.1038/nrmicro1445.
11. Smirnova GV, Oktyabrsky ON. 2018. Relationship between Escherichia coli growth rate and bacterial susceptibility to ciprofloxacin. FEMS Microbiol Lett 365:fnx254. https://doi.org/10.1093/femsle/fnx254.
12. Tuomanen E, Durack DT, Tomasz A. 1986. Antibiotic tolerance among clinical isolates of bacteria. Antimicrob Agents Chemother 30:521–527. https://doi.org/10.1128/aac.30.4.521.
13. Liu J, Gefen O, Ronin I, Bar-Meir M, Balaban NQ. 2020. Effect of tolerance on the evolution of antibiotic resistance under drug combinations. Science 367:200–204. https://doi.org/10.1126/science.aay3041.
14. Tuomanen E, Cozens R, Tosch W, Zak O, Tomasz A. 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. https://doi.org/10.1099/00221287-132-5-1297.
15. Casadesús J, Low DA. 2013. Programmed heterogeneity: epigenetic mechanisms in bacteria. J Biol Chem 288:13929–13935. https://doi.org/10.1074/jbc.R113.472274.
16. Davis KM, Isberg RR. 2016. Defining heterogeneity within bacterial populations via single cell approaches. Bioessays 38:782–790. https://doi.org/10.1002/bies.201500121.
17. Lopez D, Vlamakis H, Kolter R. 2009. Generation of multiple cell types in *Bacillus subtilis*. FEMS Microbiol Rev 33:152–163. https://doi.org/10.1111/j.1574-6976.2008.00148.x.
18. Sánchez-Romero MA, Casadesús J. 2020. The bacterial epigenome. Nat Rev Microbiol 18:7–20. https://doi.org/10.1038/s41579-019-0286-2.
19. Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S. 2004. Bacterial persistence as a phenotypic switch. Science 305:1622–1625. https://doi.org/10.1126/science.1099390.
20. 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. https://doi.org/10.1126/sciadv.aav9462.
21. Helaine S, Cheverton AM, Watson KG, Faure LM, Matthews SA, Holden DW. 2014. Internalization of Salmonella by macrophages induces formation of nonreplicating persisters. Science 343:204–208. https://doi.org/10.1126/science.1244705.
22. 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 158:722–733. https://doi.org/10.1016/j.cell.2014.06.045.
23. Joers A, Kaldalu N, Tenson T. 2010. The frequency of persisters in Escherichia coli reflects the kinetics of awakening from dormancy. J Bacteriol 192:3379–3384. https://doi.org/10.1128/JB.00056-10.
24. Setlow P. 2014. Spore resistance properties. Microbiol Spectr 2:TBS-0003-2012. https://doi.org/10.1128/microbiolspec.TBS-0003-2012.
25. Roostalu J, Jõers A, Luidalepp H, Kaldalu N, Tenson T. 2008. Cell division in Escherichia coli cultures monitored at single cell resolution. BMC Microbiol 8:68. https://doi.org/10.1186/1471-2180-8-68.
26. Völzing KG, Brynildsen MP. 2015. Stationary-phase persisters to ofloxacin sustain DNA damage and require repair systems only during recovery. mBio 6:e00731-15. https://doi.org/10.1128/mBio.00731-15.
27. Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K. 2004. Persister cells and tolerance to antimicrobials. FEMS Microbiol Lett 230:13–18. https://doi.org/10.1016/S0378-1097(03)00856-5.
28. Bigger J. 1944. Treatment of staphylococcal infections with penicillin by intermittent sterilization. Lancet 244:497–500. https://doi.org/10.1016/S0140-6736(00)74210-3.
29. Del Pozo JL. 2018. Biofilm-related disease. Expert Rev Anti Infect Ther 16:51–65. https://doi.org/10.1080/14787210.2018.1417036.
30. Conlon BP, Rowe SE, Lewis K. 2015. Persister cells in biofilm associated infections. Adv Exp Med Biol 831:1–9. https://doi.org/10.1007/978-3-319-09782-4_1.
31. Gold B, Nathan C. 2017. Targeting phenotypically tolerant Mycobacterium tuberculosis. Microbiol Spectr 5:TBTB2-0031-2016. https://doi.org/10.1128/microbiolspec.TBTB2-0031-2016.
32. Ejrnæs K. 2011. Bacterial characteristics of importance for recurrent urinary tract infections caused by Escherichia coli. Dan Med Bull 58:B4187.
33. 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. https://doi.org/10.1128/JB.01651-09.
34. Levin-Reisman I, Ronin I, Gefen O, Braniss I, Shoresh N, Balaban NQ. 2017. Antibiotic tolerance facilitates the evolution of resistance. Science 355:826–830. https://doi.org/10.1126/science.aaj2191.
35. Levin-Reisman I, Brauner A, Ronin I, Balaban NQ. 2019. Epistasis between antibiotic tolerance, persistence, and resistance mutations. Proc Natl Acad Sci U S A 116:14734–14739. https://doi.org/10.1073/pnas.1906169116.
36. Windels EM, Michiels JE, Fauvart M, Wenseleers T, Van den Bergh B, Michiels J. 2019. Bacterial persistence promotes the evolution of antibiotic resistance by increasing survival and mutation rates. ISME J 13:1239–1251. https://doi.org/10.1038/s41396-019-0344-9.
37. 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. https://doi.org/10.1038/s41467-019-09058-4.
38. Bakkeren E, Huisman JS, Fattinger SA, Hausmann A, Furter M, Egli A, Slack E, Sellin ME, Bonhoeffer S, Regoes RR, Diard M, Hardt W-D. 2019. Salmonella persisters promote the spread of antibiotic resistance plasmids in the gut. Nature 573:276–280. https://doi.org/10.1038/s41586-019-1521-8.
39. Goormaghtigh F, Fraikin N, Putrinš M, Hallaert T, Hauryliuk V, Garcia-Pino A, Sjödin A, Kasvandik S, Udekwu K, Tenson T, Kaldalu N, Van Melderen L. 2018. Reassessing the role of type II toxin-antitoxin systems in formation of Escherichia coli type II persister cells. mBio 9:e00640-18. https://doi.org/10.1128/mBio.00640-18.
40. Gefen O, Gabay C, Mumcuoglu M, Engel G, Balaban NQ. 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. https://doi.org/10.1073/pnas.0711712105.
41. Yamamoto N, Isshiki R, Kawai Y, Tanaka D, Sekiguchi T, Matsumoto S, Tsuneda S. 2018. Stochastic expression of lactate dehydrogenase A induces Escherichia coli persister formation. J Biosci Bioeng 126:30–37. https://doi.org/10.1016/j.jbiosc.2018.01.016.
42. Levin BR, Concepción-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. https://doi.org/10.1016/j.mib.2014.06.016.
43. Pontes MH, Groisman EA. 2019. Slow growth determines nonheritable antibiotic resistance in *Salmonella enterica*. Sci Signal 12:eaax3938. https://doi.org/10.1126/scisignal.aax3938.
44. Conlon BP, Rowe SE, Gandt AB, Nuxoll AS, Donegan NP, Zalis EA, Clair G, Adkins JN, Cheung AL, Lewis K. 2016. Persister formation in Staphylococcus aureus is associated with ATP depletion. Nat Microbiol 1:16051. https://doi.org/10.1038/nmicrobiol.2016.51.
45. Pu Y, Li Y, Jin X, Tian T, Ma Q, Zhao Z, Lin S, Chen Z, Li B, Yao G, Leake MC, Lo C-J, Bai F. 2019. ATP-dependent dynamic protein aggregation regulates bacterial dormancy depth critical for antibiotic tolerance. Mol Cell 73:143–156.e4. https://doi.org/10.1016/j.molcel.2018.10.022.
46. Pu Y, Zhao Z, Li Y, Zou J, Ma Q, Zhao Y, Ke Y, Zhu Y, Chen H, Baker MAB, Ge H, Sun Y, Xie XS, Bai F. 2016. Enhanced efflux activity facilitates drug tolerance in dormant bacterial cells. Mol Cell 62:284–294. https://doi.org/10.1016/j.molcel.2016.03.035.
47. Shan Y, Brown Gandt A, Rowe SE, Deisinger JP, Conlon BP, Lewis K. 2017. ATP-dependent persister formation in *Escherichia coli*. mBio 8:e02267-16. https://doi.org/10.1128/mBio.02267-16.
48. Lee SW, Foley EJ, Epstein JA. 1944. Mode of action of penicillin. I. Bacterial growth and penicillin activity-Staphylococcus aureus FDA. J Bacteriol 48:393–399. https://doi.org/10.1128/JB.48.4.393-399.1944.
49. Hobby GL, Meyer K, Chaffee E. 1942. Observations on the mechanism of action of penicillin. Exp Biol Med 50:281–285. https://doi.org/10.3181/00379727-50-13773.
50. Orman MA, Henry TC, DeCoste CJ, Brynildsen MP. 2016. Analyzing persister physiology with fluorescence-activated cell sorting. Methods Mol Biol 1333:83–100. https://doi.org/10.1007/978-1-4939-2854-5_8.
51. Levin-Reisman I, Balaban NQ. 2016. Quantitative measurements of type I and type II persisters using ScanLag. Methods Mol Biol 1333:75–81. https://doi.org/10.1007/978-1-4939-2854-5_7.
52. Levin-Reisman I, Gefen O, Fridman O, Ronin I, Shwa D, Sheftel H,

Downloaded from https://journals.asm.org/journal/mmbr on 07 June 2021 by 193.40.12.11.

Balaban NQ. 2010. Automated imaging with ScanLag reveals previously undetectable bacterial growth phenotypes. Nat Methods 7:737–739. https://doi.org/10.1038/nmeth.1485.
53. Jõers A, Tenson T. 2016. Growth resumption from stationary phase reveals memory in Escherichia coli cultures. Sci Rep 6:24055. https://doi.org/10.1038/srep24055.
54. 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. https://doi.org/10.1128/JB.00085-11.
55. Wolfson JS, Hooper DC, McHugh GL, Bozza MA, Swartz MN. 1990. Mutants of Escherichia coli K-12 exhibiting reduced killing by both quinolone and beta-lactam antimicrobial agents. Antimicrob Agents Chemother 34:1938–1943. https://doi.org/10.1128/aac.34.10.1938.
56. Nicoloff H, Hjort K, Levin BR, Andersson DI. 2019. The high prevalence of antibiotic heteroresistance in pathogenic bacteria is mainly caused by gene amplification. Nat Microbiol 4:504–514. https://doi.org/10.1038/s41564-018-0342-0.
57. Band VI, Weiss DS. 2019. Heteroresistance: a cause of unexplained antibiotic treatment failure? PLoS Pathog 15:e1007726. https://doi.org/10.1371/journal.ppat.1007726.
58. Hiramatsu K, Aritaka N, Hanaki H, Kawasaki S, Hosoda Y, Hori S, Fukuchi Y, Kobayashi I. 1997. Dissemination in Japanese hospitals of strains of Staphylococcus aureus heterogeneously resistant to vancomycin. Lancet 350:1670–1673. https://doi.org/10.1016/S0140-6736(97)07324-8.
59. Moore MR, Perdreau-Remington F, Chambers HF. 2003. Vancomycin treatment failure associated with heterogeneous vancomycin-intermediate Staphylococcus aureus in a patient with endocarditis and in the rabbit model of endocarditis. Antimicrob Agents Chemother 47:1262–1266. https://doi.org/10.1128/AAC.47.4.1262-1266.2003.
60. Khatib R, Jose J, Musta A, Sharma M, Fakih MG, Johnson LB, Riederer K, Shemes S. 2011. Relevance of vancomycin-intermediate susceptibility and heteroresistance in methicillin-resistant Staphylococcus aureus bacteraemia. J Antimicrob Chemother 66:1594–1599. https://doi.org/10.1093/jac/dkr169.
61. Napier BA, Band V, Burd EM, Weiss DS. 2014. Colistin heteroresistance in Enterobacter cloacae is associated with cross-resistance to the host antimicrobial lysozyme. Antimicrob Agents Chemother 58:5594–5597. https://doi.org/10.1128/AAC.02432-14.
62. Band VI, Crispell EK, Napier BA, Herrera CM, Tharp GK, Vavikolanu K, Pohl J, Read TD, Bosinger SE, Trent MS, Burd EM, Weiss DS. 2016. Antibiotic failure mediated by a resistant subpopulation in Enterobacter cloacae. Nat Microbiol 1:16053. https://doi.org/10.1038/nmicrobiol.2016.53.
63. Wakamoto Y, Dhar N, Chait R, Schneider K, Signorino-Gelo F, Leibler S, McKinney JD. 2013. Dynamic persistence of antibiotic-stressed mycobacteria. Science 339:91–95. https://doi.org/10.1126/science.1229858.
64. Sánchez-Romero MA, Casadesús J. 2014. Contribution of phenotypic heterogeneity to adaptive antibiotic resistance. Proc Natl Acad Sci U S A 111:355–360. https://doi.org/10.1073/pnas.1316084111.
65. Elf J, Nilsson K, Tenson T, Ehrenberg M. 2006. Bistable bacterial growth rate in response to antibiotics with low membrane permeability. Phys Rev Lett 97:258104. https://doi.org/10.1103/PhysRevLett.97.258104.
66. Harms A, Fino C, Sørensen MA, Semsey S, Gerdes K. 2017. Prophages and growth dynamics confound experimental results with antibiotic-tolerant persister cells. mBio 8:e01964-17. https://doi.org/10.1128/mBio.01964-17.
67. Goormaghtigh F, Van Melderen L. 2016. Optimized method for measuring persistence in Escherichia coli with improved reproducibility. Methods Mol Biol 1333:43–52. https://doi.org/10.1007/978-1-4939-2854-5_4.
68. Amato SM, Brynildsen MP. 2015. Persister heterogeneity arising from a single metabolic stress. Curr Biol 25:2090–2098. https://doi.org/10.1016/j.cub.2015.06.034.
69. Amato SM, Brynildsen MP. 2014. Nutrient transitions are a source of persisters in Escherichia coli biofilms. PLoS One 9:e93110. https://doi.org/10.1371/journal.pone.0093110.
70. Amato SM, Orman MA, Brynildsen MP. 2013. Metabolic control of persister formation in Escherichia coli. Mol Cell 50:475–487. https://doi.org/10.1016/j.molcel.2013.04.002.
71. Kotte O, Volkmer B, Radzikowski JL, Heinemann M. 2014. Phenotypic bistability in Escherichia coli's central carbon metabolism. Mol Syst Biol 10:736. https://doi.org/10.15252/msb.20135022.
72. Radzikowski JL, Vedelaar S, Siegel D, Ortega ÁD, Schmidt A, Heinemann M. 2016. Bacterial persistence is an active σ^S stress response to metabolic flux limitation. Mol Syst Biol 12:882. https://doi.org/10.15252/msb.20166998.
73. Ankomah P, Johnson PJT, Levin BR. 2013. The Pharmaco –, population and evolutionary dynamics of multi-drug therapy: experiments with S. aureus and E. coli and computer simulations. PLoS Pathog 9:e1003300. https://doi.org/10.1371/journal.ppat.1003300.
74. Kudrin P, Varik V, Oliveira SRA, Beljantseva J, Del Peso Santos T, Dzhygyr I, Rejman D, Cava F, Tenson T, Hauryliuk V. 2017. Subinhibitory concentrations of bacteriostatic antibiotics induce relA-dependent and relA-independent tolerance to β-lactams. Antimicrob Agents Chemother 61:e02173-16. https://doi.org/10.1128/AAC.02173-16.
75. Kwan BW, Valenta JA, Benedik MJ, Wood TK. 2013. Arrested protein synthesis increases persister-like cell formation. Antimicrob Agents Chemother 57:1468–1473. https://doi.org/10.1128/AAC.02135-12.
76. 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. https://doi.org/10.1021/acs.jproteome.8b00625.
77. Ocampo PS, Lázár V, Papp B, Arnoldini M, Abel Zur Wiesch P, Busa-Fekete R, Fekete G, Pál C, Ackermann M, Bonhoeffer S. 2014. Antagonism between bacteriostatic and bactericidal antibiotics is prevalent. Antimicrob Agents Chemother 58:4573–4582. https://doi.org/10.1128/AAC.02463-14.
78. Dörr T, Lewis K, Vulić M. 2009. SOS response induces persistence to fluoroquinolones in Escherichia coli. PLoS Genet 5:e1000760. https://doi.org/10.1371/journal.pgen.1000760.
79. Dörr T, Vulić M, Lewis K. 2010. Ciprofloxacin causes persister formation by inducing the TisB toxin in Escherichia coli. PLoS Biol 8:e1000317. https://doi.org/10.1371/journal.pbio.1000317.
80. Johnson PJT, Levin BR. 2013. Pharmacodynamics, population dynamics, and the evolution of persistence in Staphylococcus aureus. PLoS Genet 9:e1003123. https://doi.org/10.1371/journal.pgen.1003123.
81. McCall IC, Shah N, Govindan A, Baquero F, Levin BR. 2019. Antibiotic killing of diversely generated populations of nonreplicating bacteria. Antimicrob Agents Chemother 63:e02360-18. https://doi.org/10.1128/AAC.02360-18.
82. Ren H, He X, Zou X, Wang G, Li S, Wu Y. 2015. Gradual increase in antibiotic concentration affects persistence of Klebsiella pneumoniae. J Antimicrob Chemother 70:3267–3272. https://doi.org/10.1093/jac/dkv251.
83. Leimer N, Rachmühl C, Palheiros Marques M, Bahlmann AS, Furrer A, Eichenseher F, Seidl K, Matt U, Loessner MJ, Schuepbach RA, Zinkernagel AS. 2016. Nonstable Staphylococcus aureus small-colony variants are induced by low pH and sensitized to antimicrobial therapy by phagolysosomal alkalinization. J Infect Dis 213:305–313. https://doi.org/10.1093/infdis/jiv388.
84. Leung V, Levesque CM. 2012. A stress-inducible quorum-sensing peptide mediates the formation of persister cells with noninherited multidrug tolerance. J Bacteriol 194:2265–2274. https://doi.org/10.1128/JB.06707-11.
85. Kubistova L, Dvoracek L, Tkadlec J, Melter O, Licha I. 2018. Environmental stress affects the formation of Staphylococcus aureus persisters tolerant to antibiotics. Microb Drug Resist 24:547–555. https://doi.org/10.1089/mdr.2017.0064.
86. Leszczynska D, Matuszewska E, Kuczynska-Wisnik D, Furmanek-Blaszk B, Laskowska E. 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. https://doi.org/10.1371/journal.pone.0054737.
87. Bhaskar A, De Piano C, Gelman E, McKinney JD, Dhar N. 2018. Elucidating the role of (p)ppGpp in mycobacterial persistence against antibiotics. IUBMB Life 70:836–844. https://doi.org/10.1002/iub.1888.
88. Mok WWK, Brynildsen MP. 2018. Timing of DNA damage responses impacts persistence to fluoroquinolones. Proc Natl Acad Sci U S A 115:E6301–E6309. https://doi.org/10.1073/pnas.1804218115.
89. Allison KR, Brynildsen MP, Collins JJ. 2011. Metabolite-enabled eradication of bacterial persisters by aminoglycosides. Nature 473:216–220. https://doi.org/10.1038/nature10069.
90. Spoering AL, Lewis K. 2001. Biofilms and planktonic cells of Pseudomonas aeruginosa have similar resistance to killing by antimicrobials. J Bacteriol 183:6746–6751. https://doi.org/10.1128/JB.183.23.6746-6751.2001.
91. Niu H, Cui P, Yee R, Shi W, Zhang S, Feng J, Sullivan D, Zhang W, Zhu B, Zhang Y. 2015. A clinical drug library screen identifies tosufloxacin as being highly active against Staphylococcus aureus persisters. Antibiotics (Basel)) 4:329–336. https://doi.org/10.3390/antibiotics4030329.

Downloaded from https://journals.asm.org/journal/mmbr on 07 June 2021 by 193.40.12.11.

92. Niu H, Cui P, Shi W, Zhang S, Feng J, Wang Y, Sullivan D, Zhang W, Zhu B, Zhang Y. 2015. Identification of anti-persister activity against uropathogenic Escherichia coli from a clinical drug library. Antibiotics (Basel)) 4:179–187. https://doi.org/10.3390/antibiotics4020179.
93. Lindner AB, Madden R, Demarez A, Stewart EJ, Taddei F. 2008. Asymmetric segregation of protein aggregates is associated with cellular aging and rejuvenation. Proc Natl Acad Sci U S A 105:3076–3081. https://doi.org/10.1073/pnas.0708931105.
94. 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. https://doi.org/10.1016/j.chom.2014.11.016.
95. Stapels DAC, Hill PWS, Westermann AJ, Fisher RA, Thurston TL, Saliba AE, Blommestein I, Vogel J, Helaine S. 2018. Salmonella persisters undermine host immune defenses during antibiotic treatment. Science 362:1156–1160. https://doi.org/10.1126/science.aat7148.
96. Peyrusson F, Varet H, Nguyen TK, Legendre R, Sismeiro O, Coppée JY, Wolz C, Tenson T, Van Bambeke F. 2020. Intracellular Staphylococcus aureus persisters upon antibiotic exposure. Nat Commun 11:1–14. https://doi.org/10.1038/s41467-020-15966-7.
97. Losick R, Youngman P, Piggot PJ. 1986. Genetics of endospore formation in Bacillus subtilis. Annu Rev Genet 20:625–669. https://doi.org/10.1146/annurev.ge.20.120186.003205.
98. 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. https://doi.org/10.1128/AAC.00052-07.
99. 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. https://doi.org/10.1016/j.femsle.2004.11.049.
100. 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. https://doi.org/10.1128/AAC.00144-08.
101. 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. https://doi.org/10.1128/JB.155.2.768-775.1983.
102. Scherrer R, Moyed HS. 1988. Conditional impairment of cell division and altered lethality in hipA mutants of Escherichia coli K-12. J Bacteriol 170:3321–3326. https://doi.org/10.1128/jb.170.8.3321-3326.1988.
103. Goneau LW, Yeoh NS, MacDonald KW, Cadieux PA, Burton JP, Razvi H, Reid G. 2014. Selective target inactivation rather than global metabolic dormancy causes antibiotic tolerance in uropathogens. Antimicrob Agents Chemother 58:2089–2097. https://doi.org/10.1128/AAC.02552-13.
104. Black DS, Irwin B, Moyed HS. 1994. Autoregulation of hip, an operon that affects lethality due to inhibition of peptidoglycan or DNA synthesis. J Bacteriol 176:4081–4091. https://doi.org/10.1128/jb.176.13.4081-4091.1994.
105. Black DS, Kelly AJ, Mardis MJ, Moyed HS. 1991. Structure and organization of hip, an operon that affects lethality due to inhibition of peptidoglycan or DNA synthesis. J Bacteriol 173:5732–5739. https://doi.org/10.1128/jb.173.18.5732-5739.1991.
106. Germain E, Castro-Roa D, Zenkin N, Gerdes K. 2013. Molecular mechanism of bacterial persistence by HipA. Mol Cell 52:248–254. https://doi.org/10.1016/j.molcel.2013.08.045.
107. Kaspy I, Rotem E, Weiss N, Ronin I, Balaban NQ, Glaser G. 2013. HipA-mediated antibiotic persistence via phosphorylation of the glutamyl-tRNA-synthetase. Nat Commun 4:3001. https://doi.org/10.1038/ncomms4001.
108. Schumacher MA, Balani P, Min J, Chinnam NB, Hansen S, Vulić M, Lewis K, Brennan RG. 2015. HipBA-promoter structures reveal the basis of heritable multidrug tolerance. Nature 524:59–66. https://doi.org/10.1038/nature14662.
109. Semanjski M, Germain E, Bratl K, Kiessling A, Gerdes K, Macek B. 2018. The kinases HipA and HipA7 phosphorylate different substrate pools in *Escherichia coli* to promote multidrug tolerance. Sci Signal 11:eaat5750. https://doi.org/10.1126/scisignal.aat5750.
110. Harms A, Brodersen DE, Mitarai N, Gerdes K. 2018. Toxins, targets, and triggers: an overview of toxin-antitoxin biology. Mol Cell 70:768–784. https://doi.org/10.1016/j.molcel.2018.01.003.
111. Gerdes K, Rasmussen PB, Molin S. 1986. Unique type of plasmid maintenance function: postsegregational killing of plasmid-free cells. Proc Natl Acad Sci U S A 83:3116–3120. https://doi.org/10.1073/pnas.83.10.3116.
112. Tsilibaris V, Maenhaut-Michel G, Mine N, Van Melderen L. 2007. What is the benefit to Escherichia coli of having multiple toxin-antitoxin systems in its genome? J Bacteriol 189:6101–6108. https://doi.org/10.1128/JB.00527-07.
113. Keren I, Minami S, Rubin E, Lewis K. 2011. Characterization and transcriptome analysis of Mycobacterium tuberculosis persisters. mBio 2:e00100-11. https://doi.org/10.1128/mBio.00100-11.
114. Shah D, Zhang Z, Khodursky A, Kaldalu N, Kurg K, Lewis K. 2006. Persisters: a distinct physiological state of E. coli. BMC Microbiol 6:53. https://doi.org/10.1186/1471-2180-6-53.
115. LeRoux M, Culviner PH, Liu YJ, Littlehale ML, Laub MT. 2020. Stress can induce transcription of toxin-antitoxin systems without activating toxin. Mol Cell 79:280–292.e8. https://doi.org/10.1016/j.molcel.2020.05.028.
116. Maisonneuve E, Shakespeare LJ, Jørgensen MG, Gerdes K. 2011. Bacterial persistence by RNA endonucleases. Proc Natl Acad Sci U S A 108:13206–13211. https://doi.org/10.1073/pnas.1100186108.
117. Maisonneuve E, Castro-Camargo M, Gerdes K. 2013. (p)ppGpp controls bacterial persistence by stochastic induction of toxin-antitoxin activity. Cell 154:1140–1150. https://doi.org/10.1016/j.cell.2013.07.048.
118. Harms A, Maisonneuve E, Gerdes K. 2016. Mechanisms of bacterial persistence during stress and antibiotic exposure. Science 354:aaf4268. https://doi.org/10.1126/science.aaf4268.
119. Maisonneuve E, Gerdes K. 2014. Molecular mechanisms underlying bacterial persisters. Cell 157:539–548. https://doi.org/10.1016/j.cell.2014.02.050.
120. Butt A, Higman VA, Williams C, Crump MP, Hemsley CM, Harmer N, Titball RW. 2014. The HicA toxin from *Burkholderia pseudomallei* has a role in persister cell formation. Biochem J 459:333–344. https://doi.org/10.1042/BJ20140073.
121. Curtis T, Takeuchi I, Gram L, Knudsen G. 2017. The influence of the toxin/antitoxin mazEF on growth and survival of Listeria monocytogenes under stress. Toxins (Basel) 9:31. https://doi.org/10.3390/toxins9010031.
122. Germain E, Roghanian M, Gerdes K, Maisonneuve E. 2015. Stochastic induction of persister cells by HipA through (p)ppGpp-mediated activation of mRNA endonucleases. Proc Natl Acad Sci U S A 112:5171–5176. https://doi.org/10.1073/pnas.1423536112.
123. Li M, Long Y, Liu Y, Liu Y, Chen R, Shi J, Zhang L, Jin Y, Yang L, Bai F, Jin S, Cheng Z, Wu W. 2016. HigB of Pseudomonas aeruginosa enhances killing of phagocytes by up-regulating the type III secretion system in ciprofloxacin induced persister cells. Front Cell Infect Microbiol 6:125. https://doi.org/10.3389/fcimb.2016.00125.
124. Ross BN, Micheva-Viteva S, Hong-Geller E, Torres AG. 2019. Evaluating the role of *Burkholderia pseudomallei* K96243 toxins BPSS0390, BPSS0395, and BPSS1584 in persistent infection. Cell Microbiol 21:e13096. https://doi.org/10.1111/cmi.13096.
125. Nieto C, Sadowy E, de la Campa AG, Hryniewicz W, Espinosa M. 2010. The relBE2Spn toxin-antitoxin system of Streptococcus pneumoniae: role in antibiotic tolerance and functional conservation in clinical isolates. PLoS One 5:e11289. https://doi.org/10.1371/journal.pone.0011289.
126. Wu X, Wang X, Drlica K, Zhao X. 2011. A toxin-antitoxin module in Bacillus subtilis can both mitigate and amplify effects of lethal stress. PLoS One 6:e23909. https://doi.org/10.1371/journal.pone.0023909.
127. 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. https://doi.org/10.1128/JB.188.10.3494-3497.2006.
128. Ramisetty BCM, Ghosh D, Roy Chowdhury M, Santhosh RS. 2016. What is the link between stringent response, endoribonuclease encoding type II toxin–antitoxin systems and persistence? Front Microbiol 7:1882. https://doi.org/10.3389/fmicb.2016.01882.
129. Sun C, Guo Y, Tang K, Wen Z, Li B, Zeng Z, Wang X. 2017. MqsR/MqsA toxin/antitoxin system regulates persistence and biofilm formation in Pseudomonas putida KT2440. Front Microbiol 8:840. https://doi.org/10.3389/fmicb.2017.00840.
130. Gupta K, Tripathi A, Sahu A, Varadarajan R. 2017. Contribution of the chromosomal *ccdAB* operon to bacterial drug tolerance. J Bacteriol 199:e00397-17. https://doi.org/10.1128/JB.00397-17.
131. Falla TJ, Chopra I. 1998. Joint tolerance to beta-lactam and fluoroquinolone antibiotics in Escherichia coli results from overexpression of hipA. Antimicrob Agents Chemother 42:3282–3284. https://doi.org/10.1128/AAC.42.12.3282.

Downloaded from https://journals.asm.org/journal/mmbr on 07 June 2021 by 193.40.12.11.

132. Harrison JJ, Wade WD, Akierman S, Vacchi-Suzzi C, Stremick CA, Turner RJ, Ceri H. 2009. The chromosomal toxin gene yafQ is a determinant of multidrug tolerance for Escherichia coli growing in a biofilm. Antimicrob Agents Chemother 53:2253–2258. https://doi.org/10.1128/AAC.00043-09.
133. Ma D, Mandell JB, Donegan NP, Cheung AL, Ma W, Rothenberger S, Shanks RMQ, Richardson AR, Urish KL. 2019. The toxin-antitoxin MazEF drives Staphylococcus aureus biofilm formation, antibiotic tolerance, and chronic infection. mBio 10:e01658-19. https://doi.org/10.1128/mBio.01658-19.
134. Norton JP, Mulvey MA. 2012. Toxin-antitoxin systems are important for niche-specific colonization and stress resistance of uropathogenic Escherichia coli. PLoS Pathog 8:e1002954. https://doi.org/10.1371/journal.ppat.1002954.
135. Slattery A, Victorsen AH, Brown A, Hillman K, Phillips GJ. 2013. Isolation of highly persistent mutants of Salmonella enterica serovar typhimurium reveals a new toxin-antitoxin module. J Bacteriol 195:647–657. https://doi.org/10.1128/JB.01397-12.
136. Silva-Herzog E, McDonald EM, Crooks AL, Detweiler CS. 2015. Physiologic stresses reveal a Salmonella persister state and TA family toxins modulate tolerance to these stresses. PLoS One 10:e0141343. https://doi.org/10.1371/journal.pone.0141343.
137. Cheverton AM, Gollan B, Przydacz M, Wong CT, Mylona A, Hare SA, Helaine S. 2016. A Salmonella toxin promotes persister formation through acetylation of tRNA. Mol Cell 63:86–96. https://doi.org/10.1016/j.molcel.2016.05.002.
138. Kaldalu N, Jõers A, Ingelman H, Tenson T. 2016. A general method for measuring persister levels in Escherichia coli cultures. Methods Mol Biol 1333:29–42. https://doi.org/10.1007/978-1-4939-2854-5_3.
139. Berghoff BA, Wagner EGH. 2017. RNA-based regulation in type I toxin-antitoxin systems and its implication for bacterial persistence. Curr Genet 63:1011–1016. https://doi.org/10.1007/s00294-017-0710-y.
140. 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. https://doi.org/10.1111/mmi.13607.
141. Verstraeten N, Knapen WJ, Kint CI, Liebens V, Van den Bergh B, Dewachter L, Michiels JE, Fu Q, David CC, Fierro AC, Marchal K, Beirlant J, Versées 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. https://doi.org/10.1016/j.molcel.2015.05.011.
142. Wilmaerts D, Dewachter L, De Loose PJ, Bollen C, Verstraeten N, Michiels J. 2019. HokB monomerization and membrane repolarization control persister awakening. Mol Cell 75:1031–1042.e4. https://doi.org/10.1016/j.molcel.2019.06.015.
143. Nguyen D, Joshi-Datar A, Lepine F, Bauerle E, Olakanmi O, Beer K, McKay G, Siehnel R, Schafhauser J, Wang Y, Britigan BE, Singh PK. 2011. Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. Science 334:982–986. https://doi.org/10.1126/science.1211037.
144. Krásny L, Gourse RL. 2004. An alternative strategy for bacterial ribosome synthesis: Bacillus subtilis rRNA transcription regulation. EMBO J 23:4473–4483. https://doi.org/10.1038/sj.emboj.7600423.
145. Wang B, Dai P, Ding D, Del Rosario A, Grant RA, Pentelute BL, Laub MT. 2019. Affinity-based capture and identification of protein effectors of the growth regulator ppGpp. Nat Chem Biol 15:141–150. https://doi.org/10.1038/s41589-018-0183-4.
146. Zhang Y, Zborníková E, Rejman D, Gerdes K. 2018. Novel (p)ppGpp binding and metabolizing proteins of Escherichia coli. mBio 9:e02188-17. https://doi.org/10.1128/mBio.02188-17.
147. Hauryliuk V, Atkinson GC, Murakami KS, Tenson T, Gerdes K. 2015. Recent functional insights into the role of (p)ppGpp in bacterial physiology. Nat Rev Microbiol 13:298–309. https://doi.org/10.1038/nrmicro3448.
148. Traxler MF, Zacharia VM, Marquardt S, Summers SM, Nguyen HT, Stark SE, Conway T. 2011. Discretely calibrated regulatory loops controlled by ppGpp partition gene induction across the "feast to famine" gradient in Escherichia coli. Mol Microbiol 79:830–845. https://doi.org/10.1111/j.1365-2958.2010.07498.x.
149. Zhu M, Pan Y, Dai X. 2019. (p)ppGpp: the magic governor of bacterial growth economy. Curr Genet 65:1121–1125. https://doi.org/10.1007/s00294-019-00973-z.
150. Potrykus K, Murphy H, Philippe N, Cashel M. 2011. ppGpp is the major source of growth rate control in E. coli. Environ Microbiol 13:563–575. https://doi.org/10.1111/j.1462-2920.2010.02357.x.
151. Bennison DJ, Irving SE, Corrigan RM. 2019. The impact of the stringent response on TRAFAC GTPases and prokaryotic ribosome assembly. Cells 8:1313. https://doi.org/10.3390/cells8111313.
152. Burgos HL, O'Connor K, Sanchez-Vazquez P, Gourse RL. 2017. Roles of transcriptional and translational control mechanisms in regulation of ribosomal protein synthesis in Escherichia coli. J Bacteriol 199:e00407-17. https://doi.org/10.1128/JB.00407-17.
153. Ross W, Sanchez-Vazquez P, Chen AY, Lee JH, Burgos HL, Gourse RL. 2016. PpGpp binding to a site at the RNAP-DksA interface accounts for its dramatic effects on transcription initiation during the stringent response. Mol Cell 62:811–823. https://doi.org/10.1016/j.molcel.2016.04.029.
154. Murray HD, Schneider DA, Gourse RL. 2003. Control of rRNA expression by small molecules is dynamic and nonredundant. Mol Cell 12:125–134. https://doi.org/10.1016/s1097-2765(03)00266-1.
155. Izutsu K, Wada A, Wada C. 2001. Expression of ribosome modulation factor (RMF) in Escherichia coli requires ppGpp. Genes Cells 6:665–676. https://doi.org/10.1046/j.1365-2443.2001.00457.x.
156. Yoshida H, Wada A. 2014. The 100S ribosome: ribosomal hibernation induced by stress. Wiley Interdiscip Rev RNA 5:723–732. https://doi.org/10.1002/wrna.1242.
157. Boehm A, Steiner S, Zaehringer F, Casanova A, Hamburger F, Ritz D, Keck W, Ackermann M, Schirmer T, Jenal U. 2009. Second messenger signalling governs Escherichia coli biofilm induction upon ribosomal stress. Mol Microbiol 72:1500–1516. https://doi.org/10.1111/j.1365-2958.2009.06739.x.
158. Chávez de Paz LE, Lemos JA, Wickström C, Sedgley CM. 2012. Role of (p)ppGpp in biofilm formation by Enterococcus faecalis. Appl Environ Microbiol 78:1627–1630. https://doi.org/10.1128/AEM.07036-11.
159. He H, Cooper JN, Mishra A, Raskin DM. 2012. Stringent response regulation of biofilm formation in vibrio cholerae. J Bacteriol 194:2962–2972. https://doi.org/10.1128/JB.00014-12.
160. Fiil N, Friesen JD. 1968. Isolation of "relaxed" mutants of Escherichia coli. J Bacteriol 95:729–731. https://doi.org/10.1128/JB.95.2.729-731.1968.
161. Lederberg J, Zinder N. 1948. Concentration of biochemical mutants of bacteria with penicillin. J Am Chem Soc 70:4267. https://doi.org/10.1021/ja01192a521.
162. Davis BD. 1948. Isolation of biochemically deficient mutants of bacteria by penicillin. J Am Chem Soc 70:4267. https://doi.org/10.1021/ja01192a520.
163. Irving SE, Corrigan RM. 2018. Triggering the stringent response: signals responsible for activating (p)ppGpp synthesis in bacteria. Microbiology (Reading) 164:268–276. https://doi.org/10.1099/mic.0.000621.
164. Liu K, Bittner AN, Wang JD. 2015. Diversity in (p)ppGpp metabolism and effectors. Curr Opin Microbiol 24:72–79. https://doi.org/10.1016/j.mib.2015.01.012.
165. Potrykus K, Cashel M. 2008. (p)ppGpp: still magical? Annu Rev Microbiol 62:35–51. https://doi.org/10.1146/annurev.micro.62.081307.162903.
166. Svenningsen MS, Veress A, Harms A, Mitarai N, Semsey S. 2019. Birth and resuscitation of (p)ppGpp induced antibiotic tolerant persister cells. Sci Rep 9:6056. https://doi.org/10.1038/s41598-019-42403-7.
167. Varik V, Oliveira SRA, Hauryliuk V, Tenson T. 2016. Composition of the outgrowth medium modulates wake-up kinetics and ampicillin sensitivity of stringent and relaxed Escherichia coli. Sci Rep 6:22308. https://doi.org/10.1038/srep22308.
168. Edlin G, Broda P. 1968. Physiology and genetics of the "ribonucleic acid control" locus in Escherichia coli. Bacteriol Rev 32:206–226. https://doi.org/10.1128/MMBR.32.3.206-226.1968.
169. Bokinsky G, Baidoo EEK, Akella S, Burd H, Weaver D, Alonso-Gutierrez J, Garcia-Martin H, Lee TS, Keasling JD. 2013. HipA-triggered growth arrest and -lactam tolerance in Escherichia coli are mediated by RelA-dependent ppGpp synthesis. J Bacteriol 195:3173–3182. https://doi.org/10.1128/JB.02210-12.
170. Jimmy S, Saha CK, Kurata T, Stavropoulos C, Oliveira SRA, Koh A, Cepauskas A, Takada H, Rejman D, Tenson T, Strahl H, Garcia-Pino A, Hauryliuk V, Atkinson GC. 2020. A widespread toxin-antitoxin system exploiting growth control via alarmone signaling. Proc Natl Acad Sci U S A 117:10500–10510. https://doi.org/10.1073/pnas.1916617117.
171. Balleza E, Kim JM, Cluzel P. 2018. Systematic characterization of maturation time of fluorescent proteins in living cells. Nat Methods 15:47–51. https://doi.org/10.1038/nmeth.4509.
172. Sherlock ME, Sudarsan N, Breaker RR. 2018. Riboswitches for the alarmone ppGpp expand the collection of RNA-based signaling systems. Proc Natl Acad Sci U S A 115:6052–6057. https://doi.org/10.1073/pnas.1720406115.
173. Dordel J, Kim C, Chung M, de la Gándara MP, Holden MTJ, Parkhill J, de

Downloaded from https://journals.asm.org/journal/mmbr on 07 June 2021 by 193.40.12.11.

Lencastre H, Bentley SD, Tomasz A. 2014. Novel determinants of antibiotic resistance: identification of mutated loci in highly methicillin-resistant subpopulations of methicillin-resistant Staphylococcus aureus. mBio 5:e01000-13. https://doi.org/10.1128/mBio.01000-13.
174. Honsa ES, Cooper VS, Mhaissen MN, Frank M, Shaker J, Iverson A, Rubnitz J, Hayden RT, Lee RE, Rock CO, Tuomanen EI, Wolf J, Rosch JW. 2017. Rela mutant enterococcus faecium with multiantibiotic tolerance arising in an immunocompromised host. mBio 8:e02124-16. https://doi.org/10.1128/mBio.02124-16.
175. 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. https://doi.org/10.1073/pnas.1205124109.
176. Spoering AL, Vulic M, Lewis K. 2006. GlpD and PlsB participate in persister cell formation in Escherichia coli. J Bacteriol 188:5136–5144. https://doi.org/10.1128/JB.00369-06.
177. Zalis EA, Nuxoll AS, Manuse S, Clair G, Radlinski LC, Conlon BP, Adkins J, Lewis K. 2019. Stochastic variation in expression of the tricarboxylic acid cycle produces persister cells. mBio 10 10:e01930-19. https://doi.org/10.1128/mBio.01930-19.
178. Van den Bergh B, Michiels JE, Wenseleers T, Windels EM, Vanden Boer P, Kestemont D, De Meester L, Verstrepen KJ, Verstraeten N, Fauvart M, Michiels J. 2016. Frequency of antibiotic application drives rapid evolutionary adaptation of Escherichia coli persistence. Nat Microbiol 1:16020. https://doi.org/10.1038/nmicrobiol.2016.20.
179. Nyström T. 2003. Conditional senescence in bacteria: death of the immortals. Mol Microbiol 48:17–23. https://doi.org/10.1046/j.1365-2958.2003.03385.x.
180. Defraine V, Fauvart M, Michiels J. 2018. Fighting bacterial persistence: current and emerging anti-persister strategies and therapeutics. Drug Resist Updat 38:12–26. https://doi.org/10.1016/j.drup.2018.03.002.
181. Kim W, Escobar I, Fuchs BB, Mylonakis E. 2019. Antimicrobial drug discovery against persisters, p 273–295. *In* Persister Cells and Infectious Disease. Springer International Publishing, Cham, Switzerland.
182. Kwan BW, Chowdhury N, Wood TK. 2015. Combatting bacterial infections by killing persister cells with mitomycin C. Environ Microbiol 17:4406–4414. https://doi.org/10.1111/1462-2920.12873.
183. Chowdhury N, Wood TL, Martínez-Vázquez M, García-Contreras R, Wood TK. 2016. DNA-crosslinker cisplatin eradicates bacterial persister cells. Biotechnol Bioeng 113:1984–1992. https://doi.org/10.1002/bit.25963.
184. Hu Y, Shamaei-Tousi A, Liu Y, Coates A. 2010. A new approach for the discovery of antibiotics by targeting non-multiplying bacteria: a novel topical antibiotic for staphylococcal infections. PLoS One 5:e11818. https://doi.org/10.1371/journal.pone.0011818.
185. Kim W, Zhu W, Hendricks GL, Van Tyne D, Steele AD, Keohane CE, Fricke N, Conery AL, Shen S, Pan W, Lee K, Rajamuthiah R, Fuchs BB, Vlahovska PM, Wuest WM, Gilmore MS, Gao H, Ausubel FM, Mylonakis E. 2018. A new class of synthetic retinoid antibiotics effective against bacterial persisters. Nature 556:103–107. https://doi.org/10.1038/nature26157.
186. Kim W, Steele AD, Zhu W, Csatary EE, Fricke N, Dekarske MM, Jayamani E, Pan W, Kwon B, Sinitsa IF, Rosen JL, Conery AL, Fuchs BB, Vlahovska PM, Ausubel FM, Gao H, Wuest WM, Mylonakis E. 2018. Discovery and optimization of nTZDpa as an antibiotic effective against bacterial persisters. ACS Infect Dis 4:1540–1545. https://doi.org/10.1021/acsinfecdis.8b00161.
187. Moreira W, Aziz DB, Dick T. 2016. Boromycin kills mycobacterial persisters without detectable resistance. Front Microbiol 7:199. https://doi.org/10.3389/fmicb.2016.00199.
188. Matsuzaki K. 2009. Control of cell selectivity of antimicrobial peptides. Biochim Biophys Acta 1788:1687–1692. https://doi.org/10.1016/j.bbamem.2008.09.013.
189. Brender JR, McHenry AJ, Ramamoorthy A. 2012. Does cholesterol play a role in the bacterial selectivity of antimicrobial peptides? Front Immunol 3:195. https://doi.org/10.3389/fimmu.2012.00195.
190. Marques CNH, Morozov A, Planzos P, Zelaya HM. 2014. The fatty acid signaling molecule cis-2-decenoic acid increases metabolic activity and reverts persister cells to an antimicrobial-susceptible state. Appl Environ Microbiol 80:6976–6991. https://doi.org/10.1128/AEM.01576-14.
191. Herisse M, Duverger Y, Martin-Verstraete I, Barras F, Ezraty B. 2017. Silver potentiates aminoglycoside toxicity by enhancing their uptake. Mol Microbiol 105:115–126. https://doi.org/10.1111/mmi.13687.
192. Kim J-S, Heo P, Yang T-J, Lee K-S, Cho D-H, Kim BT, Suh J-H, Lim H-J, Shin D, Kim S-K, Kweon D-H. 2011. Selective killing of bacterial persisters by a single chemical compound without affecting normal antibiotic-sensitive cells. Antimicrob Agents Chemother 55:5380–5383. https://doi.org/10.1128/AAC.00708-11.
193. Flentie K, Harrison GA, Tükenmez H, Livny J, Good JAD, Sarkar S, Zhu DX, Kinsella RL, Weiss LA, Solomon SD, Schene ME, Hansen MR, Cairns AG, Kulén M, Wixe T, Lindgren AEG, Chorell E, Bengtsson C, Krishnan KS, Hultgren SJ, Larsson C, Almqvist F, Stallings CL. 2019. Chemical disarming of isoniazid resistance in Mycobacterium tuberculosis. Proc Natl Acad Sci U S A 116:10510–10517. https://doi.org/10.1073/pnas.1818009116.
194. Rubinstein E. 2001. History of quinolones and their side effects. Chemotherapy 47:3–8. https://doi.org/10.1159/000057838.
195. Zheng EJ, Stokes JM, Collins JJ. 2020. Eradicating bacterial persisters with combinations of strongly and weakly metabolism-dependent antibiotics. Cell Chem Biol S2451-9456(20)30337-8. https://doi.org/10.1016/j.chembiol.2020.08.015.
196. Stokes JM, Yang K, Swanson K, Jin W, Cubillos-Ruiz A, Donghia NM, MacNair CR, French S, Carfrae LA, Bloom-Ackerman Z, Tran VM, Chiappino-Pepe A, Badran AH, Andrews IW, Chory EJ, Church GM, Brown ED, Jaakkola TS, Barzilay R, Collins JJ. 2020. A deep learning approach to antibiotic discovery. Cell 180:688–702.e13. https://doi.org/10.1016/j.cell.2020.01.021.
197. Gavrish E, Sit CS, Cao S, Kandror O, Spoering A, Peoples A, Ling L, Fetterman A, Hughes D, Bissell A, Torrey H, Akopian T, Mueller A, Epstein S, Goldberg A, Clardy J, Lewis K. 2014. Lassomycin, a ribosomally synthesized cyclic peptide, kills Mycobacterium tuberculosis by targeting the ATP-dependent protease ClpC1P1P2. Chem Biol 21:509–518. https://doi.org/10.1016/j.chembiol.2014.01.014.
198. Li DHS, Chung YS, Gloyd M, Joseph E, Ghirlando R, Wright GD, Cheng Y-Q, Maurizi MR, Guarné A, Ortega J. 2010. Acyldepsipeptide antibiotics induce the formation of a structured axial channel in ClpP: a model for the ClpX/ClpA-bound state of ClpP. Chem Biol 17:959–969. https://doi.org/10.1016/j.chembiol.2010.07.008.
199. Lee B-G, Park EY, Lee K-E, Jeon H, Sung KH, Paulsen H, Rübsamen-Schaeff H, Brötz-Oesterhelt H, Song HK. 2010. Structures of ClpP in complex with acyldepsipeptide antibiotics reveal its activation mechanism. Nat Struct Mol Biol 17:471–478. https://doi.org/10.1038/nsmb.1787.
200. Conlon BP, Nakayasu ES, Fleck LE, LaFleur MD, Isabella VM, Coleman K, Leonard SN, Smith RD, Adkins JN, Lewis K. 2013. Activated ClpP kills persisters and eradicates a chronic biofilm infection. Nature 503:365–370. https://doi.org/10.1038/nature12790.
201. Gras J. 2013. Bedaquiline for the treatment of pulmonary, multidrug-resistant tuberculosis in adults. Drugs Today (Barc) 49:353–361.
202. de Miranda Silva C, Hajihosseini A, Myrick J, Nole J, Louie A, Schmidt S, Drusano GL. 2018. Effect of linezolid plus bedaquiline against mycobacterium tuberculosis in log phase, acid phase, and nonreplicating-persister phase in an in vitro assay. Antimicrob Agents Chemother 62:e00856-18. https://doi.org/10.1128/AAC.00856-18.
203. Hu Y, Pertinez H, Liu Y, Davies G, Coates A. 2019. Bedaquiline kills persistent Mycobacterium tuberculosis with no disease relapse: an in vivo model of a potential cure. J Antimicrob Chemother 74:1627–1633. https://doi.org/10.1093/jac/dkz052.
204. Diacon AH, Pym A, Grobusch M, Patientia R, Rustomjee R, Page-Shipp L, Pistorius C, Krause R, Bogoshi M, Churchyard G, Venter A, Allen J, Palomino JC, De Marez T, van Heeswijk RPG, Lounis N, Meyvisch P, Verbeeck J, Parys W, de Beule K, Andries K, Neeley DFM. 2009. The diarylquinoline TMC207 for multidrug-resistant tuberculosis. N Engl J Med 360:2397–2405. https://doi.org/10.1056/NEJMoa0808427.
205. Olaru ID, Heyckendorf J, Andres S, Kalsdorf B, Lange C. 2017. Bedaquiline-based treatment regimen for multidrug-resistant tuberculosis. Eur Respir J 49:1700742. https://doi.org/10.1183/13993003.00742-2017.
206. Andries K, Verhasselt P, Guillemont J, Göhlmann HWH, Neefs JM, Winkler H, Van Gestel J, Timmerman P, Zhu M, Lee E, Williams P, De Chaffoy D, Huitric E, Hoffner S, Cambau E, Truffot-Pernot C, Lounis N, Jarlier V. 2005. A diarylquinoline drug active on the ATP synthase of Mycobacterium tuberculosis. Science 307:223–227. https://doi.org/10.1126/science.1106753.
207. Veziris N, Ibrahim M, Lounis N, Andries K, Jarlier V. 2011. Sterilizing activity of second-line regimens containing TMC207 in a murine model of tuberculosis. PLoS One 6:e17556. https://doi.org/10.1371/journal.pone.0017556.
208. Ibrahim M, Truffot-Pernot C, Andries K, Jarlier V, Veziris N. 2009. Sterilizing activity of R207910 (TMC207)-containing regimens in the murine

Downloaded from https://journals.asm.org/journal/mmbr on 07 June 2021 by 193.40.12.11.

model of tuberculosis. Am J Respir Crit Care Med 180:553–557. https://doi.org/10.1164/rccm.200807-1152OC.
209. McCune RM, Feldmann FM, Lambert HP, McDermott W. 1966. Microbial persistence. I. The capacity of tubercle bacilli to survive sterilization in mouse tissues. J Exp Med 123:445–468. https://doi.org/10.1084/jem.123.3.445.
210. Hu Y, Pertinez H, Ortega-Muro F, Alameda-Martin L, Liu Y, Schipani A, Davies G, Coates A. 2016. Investigation of elimination rate, persistent subpopulation removal, and relapse rates of Mycobacterium tuberculosis by using combinations of first-line drugs in a modified Cornell mouse model. Antimicrob Agents Chemother 60:4778–4785. https://doi.org/10.1128/AAC.02548-15.
211. Mukamolova GV, Turapov O, Malkin J, Woltmann G, Barer MR. 2010. Resuscitation-promoting factors reveal an occult population of tubercle bacilli in sputum. Am J Respir Crit Care Med 181:174–180. https://doi.org/10.1164/rccm.200905-0661OC.
212. Blair HA, Scott LJ. 2015. Delamanid: a review of its use in patients with multidrug-resistant tuberculosis. Drugs 75:91–100. https://doi.org/10.1007/s40265-014-0331-4.
213. Chen X, Hashizume H, Tomishige T, Nakamura I, Matsuba M, Fujiwara M, Kitamoto R, Hanaki E, Ohba Y, Matsumoto M. 2017. Delamanid kills dormant mycobacteria in vitro and in a Guinea pig model of tuberculosis. Antimicrob Agents Chemother 61:e02402-16. https://doi.org/10.1128/AAC.02402-16.
214. Lewis K. 2001. Riddle of biofilm resistance. Antimicrob Agents Chemother 45:999–1007. https://doi.org/10.1128/AAC.45.4.999-1007.2001.
215. Putrinš M, Kogermann K, Lukk E, Lippus M, Varik V, Tenson T. 2015. Phenotypic heterogeneity enables uropathogenic Escherichia coli to evade killing by antibiotics and serum complement. Infect Immun 83:1056–1067. https://doi.org/10.1128/IAI.02725-14.
216. Adams KN, Takaki K, Connolly LE, Wiedenhoft H, Winglee K, Humbert O, Edelstein PH, Cosma CL, Ramakrishnan L. 2011. Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. Cell 145:39–53. https://doi.org/10.1016/j.cell.2011.02.022.
217. Kawai Y, Mickiewicz K, Errington J. 2018. Lysozyme counteracts β-lactam antibiotics by promoting the emergence of L-form bacteria. Cell 172:1038–1049.e10. https://doi.org/10.1016/j.cell.2018.01.021.
218. Mickiewicz KM, Kawai Y, Drage L, Gomes MC, Davison F, Pickard R, Hall J, Mostowy S, Aldridge PD, Errington J. 2019. Possible role of L-form switching in recurrent urinary tract infection. Nat Commun 10:4379. https://doi.org/10.1038/s41467-019-12359-3.
219. Connolly LE, Edelstein PH, Ramakrishnan L. 2007. Why is long-term therapy required to cure tuberculosis? PLoS Med 4:e120. https://doi.org/10.1371/journal.pmed.0040120.
220. Marvig RL, Sommer LM, Molin S, Johansen HK. 2015. Convergent evolution and adaptation of Pseudomonas aeruginosa within patients with cystic fibrosis. Nat Genet 47:57–64. https://doi.org/10.1038/ng.3148.
221. Bartell JA, Sommer LM, Haagensen JAJ, Loch A, Espinosa R, Molin S, Johansen HK. 2019. Evolutionary highways to persistent bacterial infection. Nat Commun 10:629. https://doi.org/10.1038/s41467-019-08504-7.
222. Sommer LM, Marvig RL, Luján A, Koza A, Pressler T, Molin S, Johansen HK. 2016. Is genotyping of single isolates sufficient for population structure analysis of Pseudomonas aeruginosa in cystic fibrosis airways? BMC Genomics 17:589. https://doi.org/10.1186/s12864-016-2873-1.
223. Kordes A, Preusse M, Willger SD, Braubach P, Jonigk D, Haverich A, Warnecke G, Häussler S. 2019. Genetically diverse Pseudomonas aeruginosa populations display similar transcriptomic profiles in a cystic fibrosis explanted lung. Nat Commun 10:3397. https://doi.org/10.1038/s41467-019-11414-3.
224. Erdmann J, Thöming JG, Pohl S, Pich A, Lenz C, Häussler S. 2019. The core proteome of biofilm-grown clinical Pseudomonas aeruginosa isolates. Cells 8:1129. https://doi.org/10.3390/cells8101129.
225. Andersson DI, Balaban NQ, Baquero F, Courvalin P, Glaser P, Gophna U, Kishony R, Molin S, Tønjum T. 2020. Antibiotic resistance: turning evolutionary principles into clinical reality. FEMS Microbiol Rev 44:171–188. https://doi.org/10.1093/femsre/fuaa001.
226. Fernandes P. 2006. Antibacterial discovery and development: the failure of success? Nat Biotechnol 24:1497–1503. https://doi.org/10.1038/nbt1206-1497.
227. Lewis K. 2020. The science of antibiotic discovery. Cell 181:29–45. https://doi.org/10.1016/j.cell.2020.02.056.
228. El-Halfawy OM, Valvano MA. 2015. Antimicrobial heteroresistance: an emerging field in need of clarity. Clin Microbiol Rev 28:191–207. https://doi.org/10.1128/CMR.00058-14.
229. Andersson DI, Nicoloff H, Hjort K. 2019. Mechanisms and clinical relevance of bacterial heteroresistance. Nat Rev Microbiol 17:479–496. https://doi.org/10.1038/s41579-019-0218-1.
230. Drlica K, Malik M, Kerns RJ, Zhao X. 2008. Quinolone-mediated bacterial death. Antimicrob Agents Chemother 52:385–392. https://doi.org/10.1128/AAC.01617-06.
231. Serio AW, Keepers T, Andrews L, Krause KM. 2018. Aminoglycoside revival: review of a historically important class of antimicrobials undergoing rejuvenation. EcoSal Plus 8:ESP-0002-2018. https://doi.org/10.1128/ecosalplus.ESP-0002-2018.
232. Davis BD. 1987. Mechanism of bactericidal action of aminoglycosides. Microbiol Rev 51:341–350. https://doi.org/10.1128/MMBR.51.3.341-350.1987.
233. Hancock RE, Chapple DS. 1999. Peptide antibiotics. Antimicrob Agents Chemother 43:1317–1323. https://doi.org/10.1128/AAC.43.6.1317.
234. Rúben F, Amador P, Prudêncio C. 2013. β-Lactams: chemical structure, mode of action and mechanisms of resistance. Rev Med Microbiol 24:7–17. https://doi.org/10.1097/MRM.0b013e3283587727.

Niilo Kaldalu studied Biology at the University of Tartu, Estonia. He obtained his Ph.D. at the same university working with Mart Ustav on the transcriptional regulator XylS of *Pseudomonas putida* TOL plasmid. During his postdoctoral studies with Kim Lewis at Northeastern University, Boston, MA, he studied antibiotic persistence and continued the studies of antibiotic tolerance and toxin-antitoxin systems with Tanel Tenson at the University of Tartu Institute of Technology.

Vasili Hauryliuk studied chemistry at the Moscow State University, Russia (1997 to 2002). In 2008, he obtained his Ph.D. in molecular biology at Uppsala University, Sweden, working with Måns Ehrenberg on biochemical investigations of bacterial translational apparatus. Since 2008, he has been a research fellow at the University of Tartu Institute of Technology, where he is studying molecular mechanisms of protein synthesis, antibiotic resistance, and tolerance. As of 2013, he is also a group leader the Laboratory for Molecular Infection Medicine Sweden (MIMS) node of Nordic EMBL Partnership for Molecular Medicine, Umeå University. The research subjects of the Hauryliuk laboratory are bacterial ribosome quality control, (p)ppGpp-mediated stress signaling, toxin-antitoxin systems, and antibiotic resistance mediated by ABC-F ATPases.

Continued next page

Downloaded from https://journals.asm.org/journal/mmbr on 07 June 2021 by 193.40.12.11.

Kathryn Jane Turnbull studied applied biology at Northumbria University, UK before moving to Newcastle University, UK, to study for her master's degree in molecular biology. In 2017, she obtained her Ph.D. from the same university, working in the group of Kenn Gerdes on the regulation of bacterial type II toxin-antitoxin systems and bacterial stringent response. Since 2018, she has been undertaking postdoctoral research investigating (p)ppGpp-mediated signaling and ABC-F ATPase-mediated antibiotic resistance at the Laboratory for Molecular Infection Medicine Sweden (MIMS), Umeå University, in the group of Vasili Hauryliuk.

Agnese La Mensa obtained her bachelor's degree in biotechnology from the University of Urbino, Italy, and her master's degree in medical, veterinary, and forensic biotechnological sciences from the University of Perugia. During her master's studies, she completed an internship at the Department of Infectious Diseases, studying antibiotic resistance of *Escherichia coli* and *Salmonella enterica* serovar Infantis. Thereafter, she was an Erasmus Trainee at the Centre for Virus Research, MRC-University of Glasgow, UK, where she studied activation of the antiviral response in the lab of Massimo Palmarini. In 2019, she started her Ph.D. studies at the University of Tartu in Tanel Tenson's lab, focusing on chronic bacterial infections.

Marta Putrinš studied biology at the University of Tartu, Estonia. In 2008 she obtained her Ph.D. in molecular biology at the same university working with Maia Kivisaar on the phenol-dependent catabolic and stress responses in *Pseudomonas putida*. Since 2012, she has been a research fellow at the University of Tartu, Institute of Technology, working with Tanel Tenson on antibiotic tolerance and bacterial infection models.

Tanel Tenson studied biology at the University of Tartu, Estonia. He participated in a longer research exchange visit at the University of Illinois, working with Alexander Mankin. The topics studies involved the mechanisms of action of macrolide antibiotics and antibiotic resistance. The research was used for Tanel's Ph.D. thesis at University of Tartu. During postdoctoral research with Måns Ehrenberg at Uppsala University, Sweden, he studied the molecular mechanisms of bacterial protein synthesis and mechanisms of antibiotic action. He is currently working at the University of Tartu on topics related to antibiotics and antibiotic resistance.

Downloaded from https://journals.asm.org/journal/mmbr on 07 June 2021 by 193.40.12.11.