Review

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Environmental, mechanistic and evolutionary landscape of antibiotic persistence

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Abstract

Recalcitrant infections pose a serious challenge by prolonging antibiotic therapies and contributing to the spread of antibiotic resistance, thereby threatening the successful treatment of bacterial infections. One potential contributing factor in persistent infections is antibiotic persistence, which involves the survival of transiently tolerant subpopulations of bacteria. This review summarizes the current understanding of antibiotic persistence, including its clinical significance and the environmental and evolutionary factors at play. Additionally, we discuss the emerging concept of persister regrowth and potential strategies to combat persister cells. Recent advances highlight the multifaceted nature of persistence, which is controlled by deterministic and stochastic elements and shaped by genetic and environmental factors. To translate *in vitro* findings to *in vivo* settings, it is crucial to include the heterogeneity and complexity of bacterial populations in natural environments. As researchers continue to gain a more holistic understanding of this phenomenon and develop effective treatments for persistent bacterial infections, the study of antibiotic persistence is likely to become increasingly complex.

Keywords antibiotic persistence; evolution; persistent infections; persister recovery; tolerance

Subject Categories Evolution & Ecology; Microbiology, Virology & Host Pathogen Interaction

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Introduction

Bacterial species have evolved various survival strategies to deal with a myriad of stressors that impede their growth or survival (Hibbing *et al*, 2010). Among these strategies, antibiotic resistance has been well-studied and allows bacteria to thrive in antibiotic-rich environments. Despite widespread public awareness of antibiotic resistance, another critical, yet often overlooked survival strategy to antibiotics is persistence (Huemer *et al*, 2020). Antibiotic persistence is characterized by the ability of a bacterial subpopulation to tolerate a lethal antibiotic dose, while the majority of the isogenic population is rapidly killed, resulting in biphasic killing (Fig 1A). The surviving persister cells can give rise to a new population after antibiotic treatment is ceased (Balaban *et al*, 2019), provided that they have reverted to the normal, antibiotic-sensitive state and have recovered from potential antibiotic-inflicted damage (Wilmaerts *et al*, 2019b). From a clinical perspective, persistence could possibly lead to relapse of the infection, despite successful initial treatment of the patient (Fig 1B). Indeed, persistence has been linked to the chronic nature of various persistent infections (Fauvart *et al*, 2011). Moreover, the tolerance of persister cells promotes the emergence of resistance, further underpinning the importance of understanding bacterial persistence (Levin-Reisman *et al*, 2017; Windels *et al*, 2019b; Bakkeren *et al*, 2020; Santi *et al*, 2021; Sulaiman & Lam, 2021).

In the past, most research efforts have focused on investigating stochastic and deterministic persister formation *in vitro* (Van den Bergh *et al*, 2017). However, recent studies have expanded to include the mechanisms underlying persister recovery and regrowth (Wilmaerts *et al*, 2019b, 2022; Semanjski *et al*, 2021), the clinical context of the host (Helaine *et al*, 2014; Stapels *et al*, 2018; Huemer *et al*, 2021; Wang & Jin, 2022), and ecological and evolutionary aspects of bacterial persistence (Bakkeren *et al*, 2020; Personnic *et al*, 2021; Verstraete *et al*, 2022b). Therefore, there is a need for a comprehensive understanding of these recent findings. In this review, we summarize recent advances on bacterial persistence and underscore its clinical relevance. We also provide a concise overview of the genetic mechanisms underlying persistence with a focus on recovery pathways. Finally, we examine ecological and evolutionary dynamics of persistence and discuss a range of potential strategies to combat persister cells, including the usefulness of combining antibiotics and potentiating compounds.

A persistent threat in the clinic

Persistent bacterial infections pose a significant burden to human health worldwide due to their chronic nature and their challenging,

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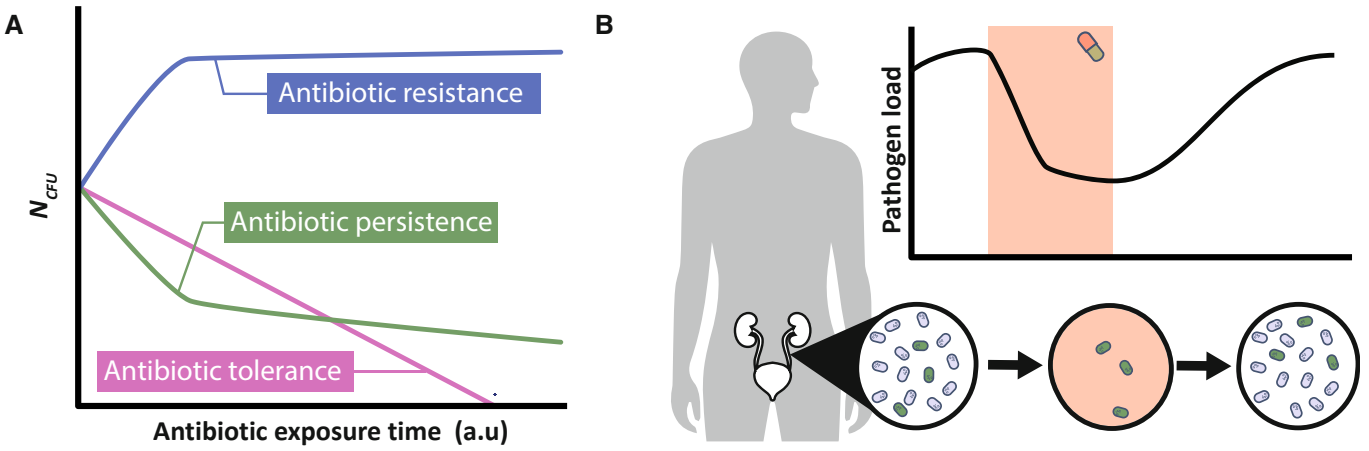

Figure 1. Distinguishing between resistance, tolerance, and persistence and the possible clinical implication of persistence.
(A) Antibiotic-resistant cells are characterized by their ability to grow during antibiotic treatment (blue). This is in contrast to antibiotic tolerance (pink) and persistence (green). In case of antibiotic tolerance, the decreased population-wide sensitivity results in slower killing, which implies that prolonged antibiotic treatment is required to eradicate the population (pink). Antibiotic persistence is a survival strategy where only a small subpopulation is highly tolerant to the antibiotic. This results in characteristic biphasic killing, where the majority of sensitive cells are rapidly killed and the subpopulation of persister cells survives. However, note that killing of persister cells can still happen at a slow rate (green). (B) A patient suffering from, for example, a urinary tract infection receives antibiotic treatment. The pathogen load in the urinary tract rapidly decreases, resulting in a seemingly successful treatment. However, once the antibiotic treatment is ceased, surviving persister cells can again increase the pathogen load, resulting in a chronic infection.

long-lasting treatment (La Rosa *et al*, 2022). Although the term "persistent infection" suggests the presence of bacterial persister cells, it rather points toward infections that are not cleared by the host immune system or, by extension, by antibiotic treatment due to antibiotic survival strategies such as resistance, tolerance and persistence (Fig 1A; Balaban *et al*, 2019). Resistance is acquired through genetic changes and denotes the ability of the bacterial population to survive and reproduce in the presence of antibiotics and is characterized by an increase in the minimum inhibitory concentration (MIC) of the antibiotic. Tolerance, on the contrary, refers to a population's ability to survive longer exposure to antibiotics, typically due to restrictive growth either caused by genetic mutations or environmental conditions. Tolerance therefore prolongs the minimum duration for killing the population, while the MIC remains unchanged since tolerant cells do not grow in the presence of antibiotics. While resistance and tolerance apply to the entire population, persistence refers to a subpopulation that consists of phenotypic variants with increased levels of tolerance. The formation of this subpopulation proceeds either deterministically, stochastically or by a combination of both. Importantly, the survival of persister cells does not affect the population's MIC (Brauner *et al*, 2016; Balaban *et al*, 2019; Ronneau *et al*, 2021). Although a fully tolerant population is characterized by a high survival rate upon treatment and the persister phenotype merely applies to a small subpopulation, persistence can have a clear fitness advantage over population-wide tolerance. While tolerance coincides with restricted growth in absence of antibiotics, a sensitive population containing a small fraction of persister cells retains colonization abilities of the host, while the persister cells ensure continuation of the infection after an antibiotic treatment (Michaux *et al*, 2022). For clarity, in the remainder of this work, the term "persistence" will be used in the context of bacterial persister cells.

The presence of antibiotic-tolerant persisters in bacterial populations can have significant implications for the success of antibiotic therapy. These cells are able to survive antibiotic exposure and then recover and regrow once the antibiotic pressure is released, causing a relapse of infection (Fig 1B; Balaban *et al*, 2019). This threat is amplified when persister cells are shielded from the host immune response, mostly by residing within biofilms (Lewis, 2007; Ciofu *et al*, 2022). Pathogens like *Pseudomonas aeruginosa*, *Staphylococcus aureus*, uropathogenic *Escherichia coli* (UPEC), and some *Salmonella* species attach to host tissues or indwelling devices, where they have been shown to form biofilms (Steenackers *et al*, 2012; Mulcahy *et al*, 2014; Speziale *et al*, 2014; Narayanan *et al*, 2018; Ciofu & Tolker-Nielsen, 2019). Apart from biofilm formation, UPEC has the ability to invade host bladder cells, thereby rendering the bacteria once again inaccessible for the host immune defense (Anderson *et al*, 2004). *Mycobacterium tuberculosis* and *Salmonella* spp., on the contrary, directly interact with the host immune system by reprogramming macrophages upon macrophage internalization. The specific intracellular conditions inside the macrophage subsequently trigger the bacteria to enter the persister state (Gengenbacher & Kaufmann, 2012; Helaine *et al*, 2014; Stapels *et al*, 2018). Research on pathogens isolated from patients with relapsing infections supports the notion that bacterial persistence plays a role in the chronic nature of these infections. For example, high-persistence (Hip) mutants were identified in clinical isolates of *P. aeruginosa*, UPEC, and *M. tuberculosis* derived from patients that underwent repeated antibiotic treatment and experienced infection relapse (Mulcahy *et al*, 2010; Schumacher *et al*, 2015; Torrey *et al*, 2016; Bartell *et al*, 2020). In addition, *S. aureus* clinical isolates from persistent infections showed increased persister levels compared with laboratory strains (Huemer *et al*, 2021), and invasive nontyphoidal *Salmonella* clinical isolates that have undergone multiple rounds of

treatment retain the characteristics of persistence rather than evolving toward tolerance (Hill *et al*, 2021). Lastly, another important threat that comes with the presence of persister cells is their ability to facilitate the emergence and spread of resistance (Levin-Reisman *et al*, 2017; Windels *et al*, 2019c; Bakkeren *et al*, 2020; Santi *et al*, 2021; Sulaiman & Lam, 2021). To fully comprehend antibiotic persistence and its implications for clinical settings, it is essential to consider environmental and evolutionary factors.

Surviving environmental adversity

Persistence is a phenotype that is heavily influenced by environmental conditions. Bacteria can form persisters in response to various stressors, such as low oxygen levels, nutrient scarcity, heat, acidity, and exposure to toxic compounds, including antibiotics (Fig 2A; Boon & Dick, 2012; Gutierrez *et al*, 2017; Wang *et al*, 2017; Kubistova *et al*, 2018; Paranjape & Shashidhar, 2019; Van den Bergh *et al*, 2022). These so-called induced or triggered persisters have been extensively studied in the laboratory and are predominantly present during stationary phase, when bacteria enter a low metabolic state to survive in a nutrient-limited environment (Verstraeten *et al*, 2015; Brown, 2019). In contrast, persisters during exponential growth or following dilution in fresh medium are relatively few (Keren *et al*, 2004; Gutierrez *et al*, 2017; Salcedo-Sora & Kell, 2020). This latter type of persisters is referred to as spontaneous persisters and are formed stochastically during steady-state exponential growth when cells are most uniform (Fig 2A; Keren *et al*, 2004; Kussell *et al*, 2005; Balaban *et al*, 2019).

The ability of bacteria to persist is not only triggered by abiotic environmental challenges but also through communication with other bacteria (Vega *et al*, 2012; Personnic *et al*, 2023). Quorum sensing offers a means for bacteria to communicate with each other using signal molecules, enabling them to induce virulence factors and coordinate behavior, examples of which are swarming or biofilm formation. In *Legionella pneumophila*, quorum sensing is a major regulator of phenotypic heterogeneity and the *Legionella* quorum sensing system controls the ratio between growing and nongrowing—antibiotic-tolerant—states (Personnic *et al*, 2021). Similarly, *Streptococcus mutans* and *P. aeruginosa* exhibit augmented antibiotic persistence attributed to the secretion of signaling molecules (Möker *et al*, 2010; Leung & Lévesque, 2012). Moreover, these signaling molecules may also trigger persistence in other species besides their own (Leung & Lévesque, 2012). These findings suggest that quorum sensing-mediated persistence may represent a collective social behavior that enables dense bacterial populations to survive hostile environments. This behavior may play a role in the formation of persisters in biofilms, which are dense microbial communities that are notoriously difficult to treat with antibiotics, with persister levels up to 1,000-fold higher than planktonic cells in exponential growth phase (Spoering & Lewis, 2001; Lewis, 2005). However, heterogenous environmental conditions, such as gradients in nutrient or oxygen availability leading to localized growth arrest, could also induce persistence within biofilms (Borriello *et al*, 2004; Nguyen *et al*, 2011; Flemming *et al*, 2016).

During bacterial infections, host immune cells employ various strategies to generate hostile conditions for pathogens. Examples include the production of reactive oxygen species (ROS), the sequestration of essential nutrients, or the release of inflammatory mediators and antimicrobial peptides (Foster, 1999; Fang, 2011; Becker & Skaar, 2014; Murdoch & Skaar, 2022). However, while these host immune-mediated stress conditions are effective in controlling

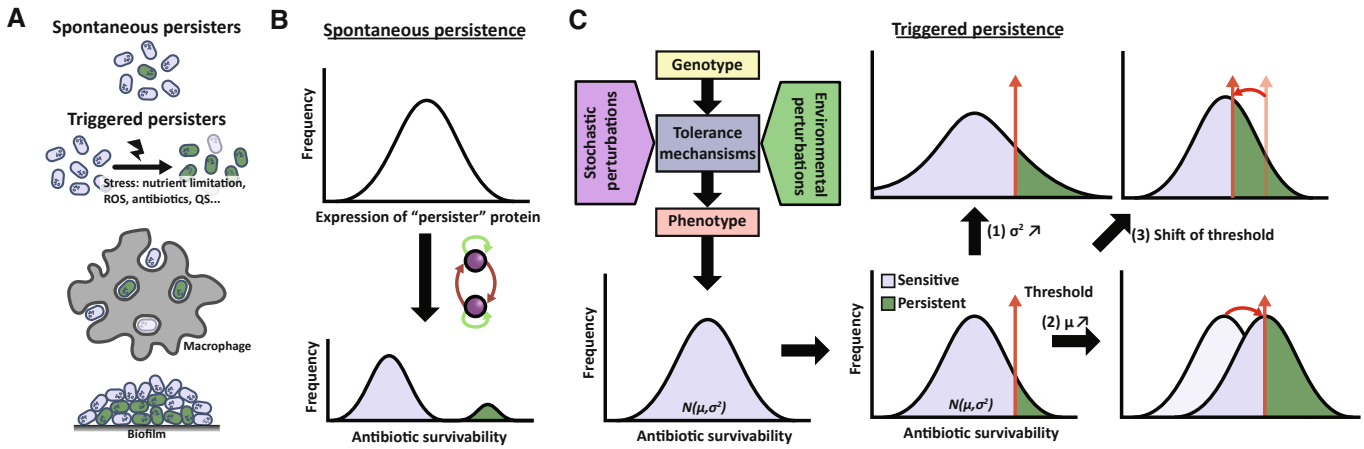

Figure 2. Formation and heterogeneity of antibiotic persisters.
(A) Two categories of persister cells are depicted: spontaneous and triggered persistence. Spontaneous persisters arise stochastically due to cellular noise, whereas triggered persisters form in response to environmental stressors, such as abiotic stress, macrophage- or biofilm-associated stresses. Abbreviations: ROS, reactive oxygen species; QS, Quorum sensing molecules. (B) and (C) present the models that explain how both types of persisters arise heterogeneously in a population. (B) Spontaneous persisters arise from random variation in persister protein expression, coupled with feedback loops, resulting in bistable phenotypic differentiation. (C) Triggered persisters, on the contrary, represent a subset of cells with the highest level of antibiotic tolerance within a population with varying levels of susceptibility arising from developmental noise in various tolerance mechanisms. The proportion of persisters depends on both the (1) mean (μ) and (2) variance (σ) of this distribution as well as the (3) specific antibiotic conditions and duration. Perturbing the mean, such as through environmental triggers or genetic mutations, can increase the fraction of cells surviving antibiotic exposure. Increasing the variance of this distribution, through environmental heterogeneity or genetic changes in buffering or potentiating genes, can also increase the fraction of survivors.

infections, they may unintentionally induce persister formation, which can negatively affect drug efficacy (Helaine *et al*, 2014; Liu *et al*, 2016; Beam *et al*, 2021). For example, macrophages induce persistence in phagocytosed *S. aureus* through the production of reactive oxygen and nitrogen species and by creating acid stress within the phagosome (Rowe *et al*, 2020; Beam *et al*, 2021; Huemer *et al*, 2021; Ronneau *et al*, 2023). In this case, persistence is likely induced through general stress responses (Peyrusson *et al*, 2020; Ranganathan *et al*, 2020). Other bacterial species, such as *Salmonella enterica* and *M. tuberculosis*, similarly display increased tolerance to antibiotics following internalization by macrophages (Helaine *et al*, 2014; Liu *et al*, 2016). Another mechanism by which the host immune system may induce bacterial persistence is through its use of antimicrobial host defense peptides, which represent an important antimicrobial component of the innate immune system (Mookherjee *et al*, 2020). However, sublethal doses of certain antimicrobial peptides prime bacterial cells and increase their tolerance and persistence (Rodríguez-Rojas *et al*, 2021; Sandín *et al*, 2022). In conclusion, pathogens face fluctuating and unfavorable conditions during infection, partly due to the host's immune response, which can paradoxically promote pathogen persistence. To combat persistent infections, it is crucial to consider the interplay between pathogenic bacteria, the host immune response, and the heterogeneous infection environment.

Heterogeneity and bacterial persistence

It is important to note that, even in cases of triggered persistence, antibiotic persistence is subject to cell-to-cell differences since per definition only a subset of the population exhibits antibiotic tolerance. The question then arises as to how this heterogeneity in antibiotic survivability in genetically uniform populations emerges. In natural environments such as in the gut or the soil, or during an infection, microbial populations are exposed to significant environmental heterogeneity (Nguyen *et al*, 2021; Sokol *et al*, 2022). Such heterogeneity within the same spatial niche is likely to result in drastic differentiation of physiological states, potentially resulting in variations in antibiotic susceptibility that may explain recalcitrant infections in some situations. Although microfluctuations or subtle environmental gradients cannot be fully precluded, well-mixed laboratory cultures generally display limited temporal and spatial variability (Junkins *et al*, 2022). However, even under these homogenous conditions, persister subpopulations can still emerge, highlighting the stochastic nature of antibiotic persistence.

In the absence of environmental or genetic variation, phenotypic heterogeneity can be attributed to stochastic cellular noise (Elowitz *et al*, 2002). The latter refers to random fluctuations in gene expression and biochemical processes within individual cells (Ackermann, 2015). This noise arises from various sources such as the inherent randomness of chemical reactions, stochastic partitioning during cell division, or cell cycle and age differences (Elowitz *et al*, 2002; Raser & O'Shea, 2005; Avery, 2006; Huh & Paulsson, 2011). Spontaneous persisters are believed to arise purely from stochastic fluctuations in gene expression via "persister" proteins that induce persister formation when expression stochastically reaches a specific threshold level (Fig 2B; Rotem *et al*, 2010; Dewachter *et al*, 2019). For instance, the expression of the toxin HipA can cause bistability of different states, allowing the bacterial cell to exist in either a susceptible or a persistent state (Balaban *et al*, 2004).

Like many biological phenomena, triggered persistence is the product of multiple parallel and interdependent processes, which are subject to their own mechanistic idiosyncrasies and triggers. Perturbing any of these processes (e.g., growth homeostasis, stress response, or membrane transport) can dramatically affect antibiotic susceptibility and persistence (Wilmaerts *et al*, 2019b). Moreover, it has been shown that these processes exhibit significant stochastic variation, leading to cell-to-cell heterogeneity (Raser & O'Shea, 2005; Ghosh *et al*, 2011; Kiviet *et al*, 2014; Amato & Brynildsen, 2015; Shan *et al*, 2017). To that end, it is likely that triggered persisters do not reflect a uniform subpopulation and may be formed through various parallel mechanisms and in response to different conditions (Fig 2C). Generally speaking, in case of antibiotic persistence, it is assumed that tolerance heterogeneity results in a distribution with two distinct phenotypic states consisting of a majority of susceptible cells and a minority of persister cells that arise via bistable switching. However, the probability distribution of individual cells' antibiotic susceptibility exhibits a monomodal Gaussian distribution (Scheler *et al*, 2020). Persistence may similarly represent a continuous quantitative trait rather than a binary persister–nonpersister state, which would be in line with it being a complex polygenic trait. This idea is related to the concept of "dormancy depth" (Pu *et al*, 2019; Bollen *et al*, 2021; Dewachter *et al*, 2021), which measures the extent of a cell's persistence through dormancy and is closely linked to its lag time. Dormancy refers to a reduced metabolic state that allows organisms to survive unfavorable conditions such as nutrient limitation, and although it can contribute to persistence, persister cells are not necessarily dormant and may use other mechanisms to survive antibiotics. Increasing dormancy depth within a population can be achieved by prolonging the stationary phase, leading to an increase in persisters and longer regrowth times (Pu *et al*, 2019), with viable but nonculturable cells representing the most extreme phenotype in that spectrum (Bollen *et al*, 2021; Dewachter *et al*, 2021). This concept could be extended beyond just persistence via dormancy toward triggered persistence and tolerance in general. In this manner, dormancy depth, along with other processes related to persistence including environmental factors, contribute to the overall phenotypic variation of a multifactorial phenotypic trait, for example, antibiotic survivability. Perturbations to this compound trait can arise from a variety of mechanisms including mutation, cellular noise, environmental fluctuations, and phenotypic plasticity, resulting in a shift in the mean or variance of this trait (Fig 2C). The persistence level of a population and the survival of individual cells then depends on the position of this distribution relative to a threshold determined by the experimental conditions in which the persistence level is measured. During antibiotic treatment, as the most susceptible cells are rapidly eliminated, the killing rate slows down, and only more tolerant cells remain. Depending on the experimental conditions and the threshold employed, this may result in a complete flattening of the killing curve. This perspective provides a useful unifying framework for understanding persistence and cell-to-cell tolerance heterogeneity and underscores the heterogeneous nature of persistence, not only between persisters and nonpersisters but also between individual persister cells.

Defense mechanisms of persister cells against antibiotics

The heterogenous nature of persisters is reflected in the variety of defense mechanisms that make them tolerant to antibiotics (Fig 3A). These mechanisms of antibiotic defense are commonly classified as either passive or active. Passive mechanisms primarily involve entering a dormant or quiescent state and thereby preventing the corrupting effects of antibiotics on vital cellular processes (Lewis, 2010), while active mechanisms involve other strategies such as decreasing intracellular antibiotic concentrations or actively preventing damage (Nguyen *et al*, 2011; Orman & Brynildsen, 2013; Pu *et al*, 2016). For a more in-depth discussion of this topic, readers are referred to other reviews (Van den Bergh *et al*, 2017; Harms, 2019; Wilmaerts *et al*, 2019b).

Passive defense of persister cells against antibiotics

Most antibiotics need an active target to exert their function (Eng *et al*, 1991), which means that bacteria can become tolerant by reducing cellular activity (Hu & Coates, 2012; Balaban *et al*, 2019). Indeed, persister cells are often considered to be tolerant because they reside in a dormant state characterized by reduced metabolic activity and a reduction of global cellular processes and growth (Balaban *et al*, 2004; Amato *et al*, 2013). Notably, the different mechanisms involved in persistence often share genetic components, making it difficult to define a single cause of persistence by dormancy. One way to lower metabolic activity and increase persistence is to deplete ATP (Fig 3B), for example, by the addition of arsenate (Conlon *et al*, 2016), the reduction of the membrane potential (Kwan *et al*, 2013), or the induction of the toxins TisB (Dörr *et al*, 2010) or HokB (Wilmaerts *et al*, 2018). Furthermore, the activity of essential cellular processes can be reduced by directly targeting important proteins involved in DNA replication (Tripathi *et al*, 2012), transcription, or translation (Fig 3B; Kwan *et al*, 2013). Several toxins from type II toxin–antitoxin modules such as HipA and TacT have been shown to specifically inhibit these processes (Korch & Hill, 2006; Cheverton *et al*, 2016). However, it is important to note that a general role of type II toxin–antitoxin modules in persistence is controversial (Goormaghtigh *et al*, 2018; Kaldalu *et al*, 2020) and that overexpression of other toxic proteins that do not belong to toxin–antitoxin modules can also induce persistence (Vázquez-Laslop *et al*, 2006). In addition to direct targeting of important proteins, persister cells have been associated with the presence of protein aggregates in which important proteins might be sequestered (Fig 3B; Pu *et al*, 2019; Yu *et al*, 2019; Dewachter *et al*, 2021; Goode *et al*, 2021). Indeed, various conditions, such as nutrient starvation, ATP depletion, heat shock and heterologous protein expression, not only increase persistence but also aggregation in the cells (Leszczynska *et al*, 2013; Mordukhova & Pan, 2014; Pu *et al*, 2019; Dewachter *et al*, 2021; Goode *et al*, 2021; Peyrusson *et al*, 2022). Moreover, adding osmolytes or buffering components to the growth medium decreases both persistence and protein aggregation (Leszczynska *et al*, 2013). Aggregates in persister cells

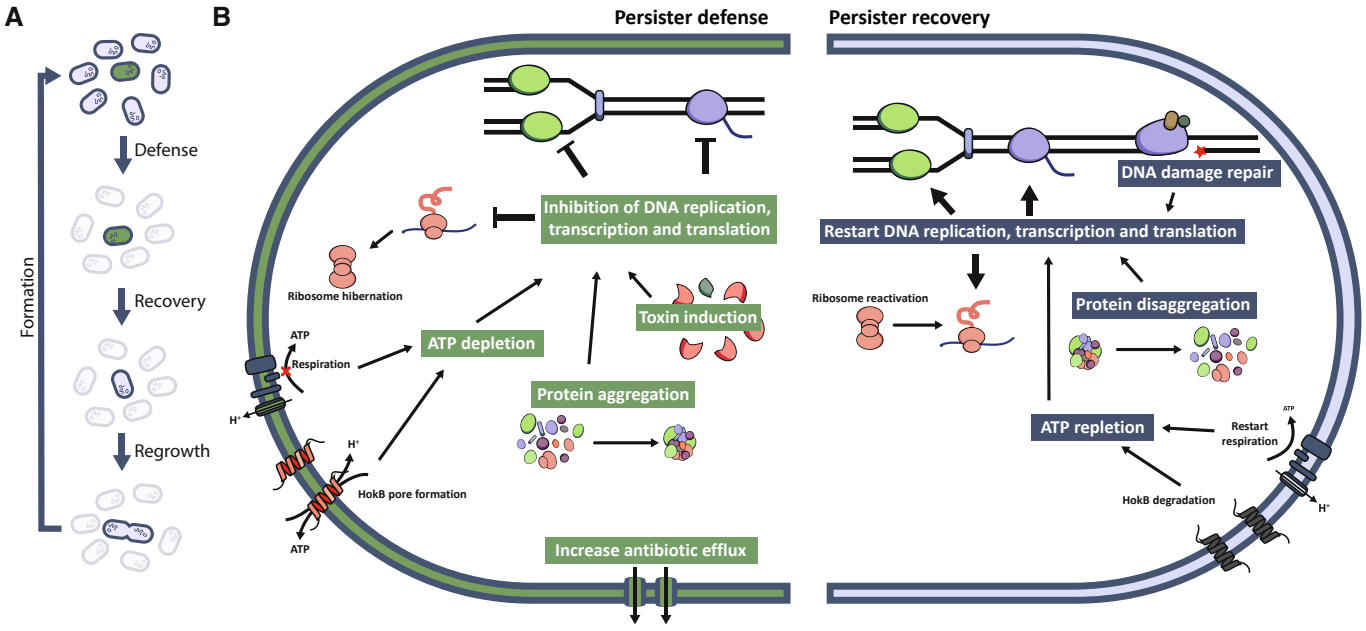

Figure 3. Mechanisms of persistence defense, recovery, and regrowth.

(A) During the course of antibiotic treatment, persister cells utilize various defense mechanisms to survive the effects of antibiotics. Once the treatment ceases, persister cells first recover from the inflicted damage before regrowing and forming a new population, where other cells can switch to the persister state. This recovery period involves repairing DNA damage and resuming critical cellular processes, such as DNA replication, transcription, and translation. (B) Persister cells can defend themselves from antibiotic damage by lowering their metabolism through inhibition of DNA replication, transcription, and translation. These pathways can be inhibited by expressing pathway-specific toxins, by sequestering essential proteins of these pathways in aggregates or by depleting the ATP needed for their functioning. Additionally, persister cells might protect themselves from more antibiotic-induced damage by increasing antibiotic efflux. For their recovery, persister cells need to repair the inflicted DNA damage. Moreover, they need to increase their metabolism to be able to regrow by restarting DNA replication, transcription and translation. To reactivate these processes, persister cells need to replete their ATP levels and remove the aggregates present in the cell.

contain many proteins that function in transcription, translation, and energy production (Pu *et al*, 2019; Dewachter *et al*, 2021; Huemer *et al*, 2021). It is therefore hypothesized that dormancy can be induced when sufficient essential proteins are contained in aggregates, thereby inhibiting the cell's overall functioning and preventing damage from antibiotics (Bollen *et al*, 2021; Dewachter *et al*, 2021).

Although tolerance of persisters to antibiotics is often attributed to their low metabolic state, dormancy alone is not sufficient to fully explain persistence. For example, there is not always a correlation between growth rate and cell survival during antibiotic treatment (Orman & Brynildsen, 2013; Wakamoto *et al*, 2013). Furthermore, some persister cells, such as intracellular persisters, still show metabolic activity and synthesize specific proteins that are important for their survival within the host cell (Orman & Brynildsen, 2013; Manina *et al*, 2015; Stapels *et al*, 2018; Wilmaerts *et al*, 2018; Peyrusson *et al*, 2020; Sulaiman & Lam, 2020; Semanjski *et al*, 2021; Mode *et al*, 2022; Ronneau *et al*, 2023). Additionally, while dormant persister cells are protected from some types of harm, they are not fully shielded from DNA damage (Völzing & Brynildsen, 2015; Wilmaerts *et al*, 2022). Moreover, it was shown that cells in stationary phase unavoidably acquire oxidative damage, the level of which is even higher in nongrowing cells (Nyström & Gustavsson, 1998; Desnues *et al*, 2003). Clearly, dormancy protects bacteria from damage to some extent, but active mechanisms are also at play.

Active defense of persister cells against antibiotics

Persister cells can actively protect themselves against antibiotics by either reducing the antibiotic concentration within the cell or by actively preventing antibiotic damage (Wilmaerts *et al*, 2019b). The former can be achieved by increasing efflux activity (Fig 3B; Pu *et al*, 2016) or preventing prodrug activation (Wakamoto *et al*, 2013). The latter can be accomplished by activating stress responses (Nguyen *et al*, 2011; Sulaiman & Lam, 2020; Semanjski *et al*, 2021; Van den Bergh *et al*, 2022). This increase in stress responses is often detected in intracellular persister cells as they need to survive exposure to a more complex environment of multiple small niches characterized by specific environmental conditions and stresses (Demarre *et al*, 2019; Peyrusson *et al*, 2020). In addition to activating stress responses, intracellular persisters have been observed to reduce the host's immune response by secreting specific effector molecules (Stapels *et al*, 2018). This indicates that intracellular persister cells use an array of mechanisms, at least part of which have already been observed in *in vitro* studies, and that they possibly depend on a combination of these mechanisms to survive in their challenging environment. This underscores the importance of verifying *in vitro* results *in vivo* (Box 1).

Box 1. In need of answers

i What is the timing and causal sequence of the processes involved in persister cell formation? To what extent are these processes independent of each other, or do they interact and influence each other in a coordinated manner?

ii What are the mechanisms of persistence *in vivo* and how do they relate to the persister mechanisms that have already been found *in vitro*?

iii In addition to repairing DNA damage, are there mechanisms in place for persister cells to repair any protein damage caused by antibiotics?

iv How do persister cells coordinate the switch from DNA repair to regrowth, and which effector proteins are involved in this process? What is the role of protein aggregates and protein synthesis in regulating this switch?

v How do persister cells respond to environmental cues, and how does this affect their recovery and proliferation? What are the clinical implications of these interactions?

vi How do environmental factors, such as nutrient availability, host immune response, and interspecies interactions, influence the evolution of persistence?

vii How do the mechanisms of persister cell regrowth and proliferation differ from those of normal bacterial growth, and how can we exploit these differences for therapeutic purposes?

viii What are the specific mechanisms underlying the efficacy of proposed antipersister compounds, and how effective are they *in vivo* in targeting persister cells? Additionally, what are the potential side effects associated with their use and can these be mitigated?

Mechanisms of persister recovery and regrowth

Persisters cells need to start recovery and regrowth to be able to recolonize the environment once antibiotics are depleted (Fig 3A). Several environmental factors can stimulate regrowth of nongrowing bacteria, including persisters, such as fresh nutrients (Jõers *et al*, 2010), quorum sensing signals from growing cells (Nichols *et al*, 2008; D'Onofrio *et al*, 2010; Jõers *et al*, 2019) and the removal of certain host immune factors (Beatty *et al*, 1993; Mohan *et al*, 2001). However, the length of the lag phase between transfer of persisters to new medium and their regrowth depends on the concentration of the antibiotic and the duration of the treatment (Himeoka & Mitarai, 2021), as well as on the intensity and length of the persistence-inducing condition (Kaplan *et al*, 2021; Cesar *et al*, 2022). Conditions of gradual stress were suggested to activate specific recovery pathways that lead to fast and homogeneous regrowth, while more acute or longer stresses induce random pathways that lead to slower and heterogeneous regrowth (Kaplan *et al*, 2021). Different mechanisms have been described to control the shift from persister to antibiotic-sensitive state, such as reprogramming of cell metabolism and damage repair (Wilmaerts *et al*, 2019b). Although these two different mechanisms will be discussed separately, a combination of both is likely necessary for persister recovery and regrowth.

Persister recovery and regrowth requires reversion to a growth-competent state

To recover from the action of the antibiotic and regrow, persisters first need to transition to a growth-competent state by replenishing their energy level and reactivating crucial cellular pathways. Indeed, persister cells were shown to increase their ATP levels before reinitiating growth (Fig 3B; Huemer *et al*, 2021; Manuse *et al*, 2021). Accordingly, nutrient-rich medium increases regrowth (Jõers *et al*, 2010; Yamasaki *et al*, 2020), and persister cells upregulate glycolysis to increase ATP levels (Semanjski *et al*, 2021). In the case of HokB-induced persisters, which are ATP-depleted as a result of membrane potential dissipation and direct ATP leaking through HokB pores (Wilmaerts *et al*, 2018), the HokB pores are degraded

and the membrane is repolarized before regrowth (Wilmaerts *et al*, 2019a). Additionally, intracellular persister cells can be locked in the persister state with low respiration and ATP levels because of intoxication of the TCA cycle by reactive nitrogen species. These locked persister cells can resume growth when the reactive nitrogen species are reduced or inhibited (Ronneau *et al*, 2023).

In addition to ATP repletion, persister cells need to reactivate essential macromolecular pathways such as DNA replication and cell division before growth is initiated (Fig 3B). During persister recovery and regrowth, the cell division genes *ftsW* and *amiA* are upregulated (Belland *et al*, 2003), while genes encoding inhibitors of cell division and DNA replication, *sulA* and *cspD*, respectively, are degraded (Langklotz & Narberhaus, 2011; Mohiuddin *et al*, 2022). Furthermore, when shifting to the growing state, persister cells increase various anabolic pathways such as the biosynthesis of multiple amino acids, secondary metabolites, and proteins (Fig 3B; Semanjski *et al*, 2021).

The increase in protein biosynthesis during persister regrowth (Semanjski *et al*, 2021) can be achieved in multiple ways. First, persister cells increase their ribosomal content and translation by increasing the production of ribosomal proteins and rRNA (Kim *et al*, 2018a; Sulaiman & Lam, 2020; Semanjski *et al*, 2021). Second, various studies suggest that persister cells reactivate inactivated ribosomes during regrowth (Yamasaki *et al*, 2020; Song & Wood, 2020a; Semanjski *et al*, 2021). Persister cells are associated with ribosomes that are inactivated by binding with the ribosome inhibitor RaiA or by ribosome dimerization (McKay & Portnoy, 2015; Prossliner *et al*, 2018; Song & Wood, 2020a). The inactivation of these ribosomes might protect them from degradation such that they can be rapidly reactivated during regrowth (Cho *et al*, 2015). Moreover, RaiA-induced ribosomes and ribosome dimers are associated with a fast and slow regrowth of stationary-phase cells, respectively. This suggests that cells have a fast and a slower mechanism for ribosome recovery after inactivation (Lang *et al*, 2021). Third, persister cells harbor many translation-related proteins in aggregates (Pu *et al*, 2019; Dewachter *et al*, 2021; Huemer *et al*, 2021). These aggregates are removed before persister regrowth with the help of chaperones DnaK and ClpB (Fig 3B; Pu *et al*, 2019; Cesar *et al*, 2022). This suggests that persister cells might use protein aggregates as temporary storage compartments from which proteins can be extracted and reused during regrowth, which is reminiscent of stress granules in quiescent eukaryotic cells (Narayanaswamy *et al*, 2009; Saad *et al*, 2017). Similarly in bacteria, FtsZ, a key cytoskeletal protein, is refolded, relocated, and reused when stationary-phase cells restart growth upon transfer to fresh medium (Yu *et al*, 2019). Finally, persister cells can increase translation by reverting the detrimental effects of translation-targeting toxins. Indeed, persister recovery and regrowth were observed following toxin inhibition by their cognate antitoxins (Pedersen *et al*, 2002; Korch & Hill, 2006; Cheverton *et al*, 2016; Rycroft *et al*, 2018) or following reversion of toxin-induced effects (Christensen *et al*, 2003; Cheverton *et al*, 2016; Rycroft *et al*, 2018).

It is important to note that some of the mechanisms of recovery and regrowth discussed above are not necessarily unique to persister cells. Stationary-phase cells also have a reduced metabolism, which requires reactivation upon transfer to fresh medium. Nevertheless, gaining more insight in these mechanisms is needed to understand how persister cells recover and regrow.

Persister recovery and regrowth depends on cellular damage repair

Besides reversion to a growth-competent state, persister cells need to repair damage, inflicted either directly or indirectly by the antibiotic or by environmental stressors, to allow recovery and regrowth (Fig 3B; Wilmaerts *et al*, 2019b).

Fluoroquinolones offer the most evident example of an antibiotic class causing direct damage to persister cells. In *E. coli*, their primary target is DNA gyrase (Drlica & Zhao, 1997; Malik *et al*, 2006), which plays a critical role in the relaxation of DNA during replication and transcription. Several studies have indicated that, following fluoroquinolone treatment, persister cells suffer DNA damage and induce the SOS response, a DNA damage-inducible DNA repair pathway, suggesting that DNA repair is essential for recovery and regrowth (Dörr *et al*, 2009; Völzing & Brynildsen, 2015; Barrett *et al*, 2019; Goormaghtigh & Van Melderen, 2019). Indeed, multiple SOS-responsive genes implicated in homologous recombination repair, including *recA*, *recB*, *ruvA*, *ruvB*, and *uvrD*, have been found to be important for persister recovery (Theodore *et al*, 2013; Völzing & Brynildsen, 2015; Mok & Brynildsen, 2018; Lemma & Brynildsen, 2021; Wilmaerts *et al*, 2022). The dependence on homologous recombination repair is further supported by the finding that persister cells often originate from cells containing a second chromosome, which may serve as a homolog in recombinational repair (Murawski & Brynildsen, 2021). While SOS induction followed by expression of SOS-responsive DNA repair genes is crucial for successful recovery from fluoroquinolone treatment, it is not considered a distinguishing factor for persistence (Mok & Brynildsen, 2018). Eventually, what distinguishes persister cells from nonpersister cells seems to be their ability to delay growth-related processes until DNA repair has been completed (Mok & Brynildsen, 2018). Indeed, a few studies using cultures in exponential growth phase point toward a mechanism of SOS-induced growth delay during fluoroquinolone treatment, either through increased production of the TisB toxin or the SulA cell division inhibitor (Dörr *et al*, 2010; Theodore *et al*, 2013; Edelmann & Berghoff, 2022).

Most bactericidal antibiotics also cause indirect damage to cells through the production of ROS (Kohanski *et al*, 2007; Foti *et al*, 2012; Dwyer *et al*, 2014; Van Acker & Coenye, 2017). The most important type of DNA damage caused by ROS is the incorporation of the mutagenic 8-oxo-guanine, formed by oxidation of the guanine nucleotide pool. While this lesion is not necessarily lethal, failed attempts to repair it prior to cell division may lead to cell death (Foti *et al*, 2012; Gruber & Walker, 2018). Successful repair of oxidative DNA damage may therefore be important for persister survival. When the level of oxidative damage is high, repair of the non-helix-distortive 8-oxo-guanine lesion is accomplished by nucleotide excision repair (NER; Gruber & Walker, 2018; Dhawale *et al*, 2021). In accordance with this, transcription-coupled NER was shown to be important for persister survival following fluoroquinolone treatment, as knockout of NER genes results in impaired persister recovery and regrowth (Wilmaerts *et al*, 2022). Transcription-coupled repair requires the removal of the RNA polymerase from the site of the lesion, either by UvrD-mediated backtracking allowing rapid resumption of transcription following repair (Epshtein *et al*, 2014), or by Mfd-mediated displacement, which terminates transcription (Park *et al*, 2002). Accordingly, UvrD-mediated backtracking was shown to result in a short persister

lag phase, whereas Mfd-mediated displacement results in a longer persister lag phase following treatment. This difference could account for lag phase heterogeneity within the persister population and enable persister cells to optimize the timing of DNA damage repair versus growth resumption (Mok & Brynildsen, 2018; Wilmaerts *et al*, 2022). Although the study of Wilmaerts *et al* (2022) focused on fluoroquinolone antibiotics, NER may also be implicated in persister survival following treatment with other classes of bactericidal antibiotics. Indeed, transcriptomics, proteomics, and transposon insertion sequencing have revealed a role for the oxidative stress response and the SOS response, including NER, during β-lactam treatment of high-persistence *hipA*-induced and *cyaA*-mutant cells (Keren *et al*, 2004; Molina-Quiroz *et al*, 2018; Sulaiman & Lam, 2020; Semanjski *et al*, 2021). However, a comprehensive study clearly linking NER of oxidative DNA damage by ROS in persister survival following treatment with β-lactam antibiotics is currently lacking.

Evolution of antibiotic persistence

All bacterial species investigated to date exhibit some level of persistence, illustrative of its universal nature. Even fungal pathogens have been observed to show persistence in response to antimicrobials (LaFleur *et al*, 2006; Bojsen *et al*, 2017). The adaptive advantage of persistence in the context of antibiotic treatment for infections is readily apparent. By investing in a diversity of phenotypes that increase their chances of long-term survival in an unpredictably changing environment, bacteria are able to hedge their bets against the possibility of encountering lethal concentrations of antibiotics (Kussell & Leibler, 2005; Grimbergen *et al*, 2015). However, the evolutionary benefits of persistence in natural environments are less straightforward as bacteria typically do not encounter such high concentrations of antibiotics in their natural ecosystems (Stepanyan *et al*, 2015). The level of persistence is determined by both genetic and environmental factors (Bakkeren *et al*, 2020; Verstraete *et al*, 2022b). Adaptive laboratory evolution has shown that antibiotic persistence is a highly evolvable trait and populations can rapidly evolve an increased persister fraction in response to periodic exposure to high antibiotic concentrations (Van den Bergh *et al*, 2016; Levin-Reisman *et al*, 2019; Windels *et al*, 2021). Given that persistence results in a reproductive advantage upon antibiotic exposure and is evolvable, it is tempting to look for an adaptive—evolutionary—explanation of antibiotic persistence. However, the mere existence of utility or function does not imply adaptation (Gould & Lewontin, 1979). As such, persistence is not necessarily an adaptation to the current environment of bacteria, but could also represent a byproduct of other adaptive—or nonadaptive—pressures that occurred in the past, that is, induction of dormancy in response to hostile environments, or a byproduct of cell-to-cell variability resulting from inherent errors in biological processes (Johnson & Levin, 2013; Levin *et al*, 2014). This nonadaptive view on persistence does not preclude it from being co-opted to increase survival in a different context (Gould & Vrba, 1982). For instance, selection may act directly on increasing persistence during adaptive laboratory evolution of persistence or within a host undergoing antibiotic treatment. However, also here, increased antibiotic persistence may have been the result of indirect or bystander selection for survival to hostile environments, provided by the immune system, rather than direct selection toward increased antibiotic tolerance (Bakkeren *et al*, 2020).

Despite the speculative nature of the evolutionary origin of persistence, the ability of persisters to survive antibiotics makes them a potential reservoir of genetic diversity that can be utilized when the environment changes, enabling the population to adapt quickly to new conditions (Windels *et al*, 2019b, 2020). In particular, the evolution of increased persistence or tolerance enables populations to evolve resistance more rapidly (Levin-Reisman *et al*, 2017). This is especially significant in conditions with high antibiotic concentrations, where enhanced tolerance allows the survival of resistance-conferring mutants beyond the mutation prevention concentration. This is facilitated through multiple mechanisms, including the increase in effective population size during antibiotic exposure, which enhances the mutation supply and increases the likelihood of acquiring resistance-conferring mutations (Levin & Rozen, 2006; Levin-Reisman *et al*, 2017), or through synergistic epistasis between tolerance and resistance mutations (Levin-Reisman *et al*, 2019). In addition, it has been found that persisters exhibit increased mutation rates, which further boost the mutational supply, enabling faster adaptation (Windels *et al*, 2019c). Persisters could also serve as a refuge for costly resistance-conferring plasmids, which can be transferred to other bacteria through horizontal gene transfer if conditions permit (Bakkeren *et al*, 2019). For a more comprehensive discussion on the evolution of persistence and its link to resistance, we encourage the reader to consult other reviews (Bakkeren *et al*, 2020; Verstraete *et al*, 2022b). Understanding how persistence evolves and affects the evolution of antibiotic resistance may ultimate allow us to develop new strategies to combat antibiotic persistence and limit resistance. Evolutionary research can aid in the development of such strategies by identifying the mechanisms and factors that promote their evolution and the interaction between persistence and resistance evolution.

Approaches for combating antibiotic persistence

As persister cells can complicate treatment and current antibiotics have proven insufficient to completely eradicate them, there is an ongoing effort to develop new antipersister strategies. While combinatorial antibiotic therapy is a promising approach (Fig 4; Keren *et al*, 2004; Aedo *et al*, 2019; Windels *et al*, 2019a), its efficacy likely varies depending on the species or strain of bacteria (Brochado *et al*, 2018), highlighting the importance of reliable identification and sensitivity profiling. However, even with optimal treatment, complete eradication of all persister cells may not be achievable due to their multiantibiotic tolerance. Therefore, there is a need for new antimicrobial compounds that can target cellular processes that are not protected in persisters (Fig 4). For example, compounds that affect cell viability by crosslinking DNA (Kwan *et al*, 2015), degrading proteins nonspecifically (Conlon *et al*, 2013) or disrupting the cell membrane (Hurdle *et al*, 2011; Defraine *et al*, 2018; Kim *et al*, 2018b; Hamad *et al*, 2022) or cell wall (Briers *et al*, 2014) can be considered. However, such antibiotics are often nonspecific and may therefore pose a risk of cytotoxic side effects. This highlights the need for further research and discovery of antimicrobial compounds that specifically target persisters and not host cells.

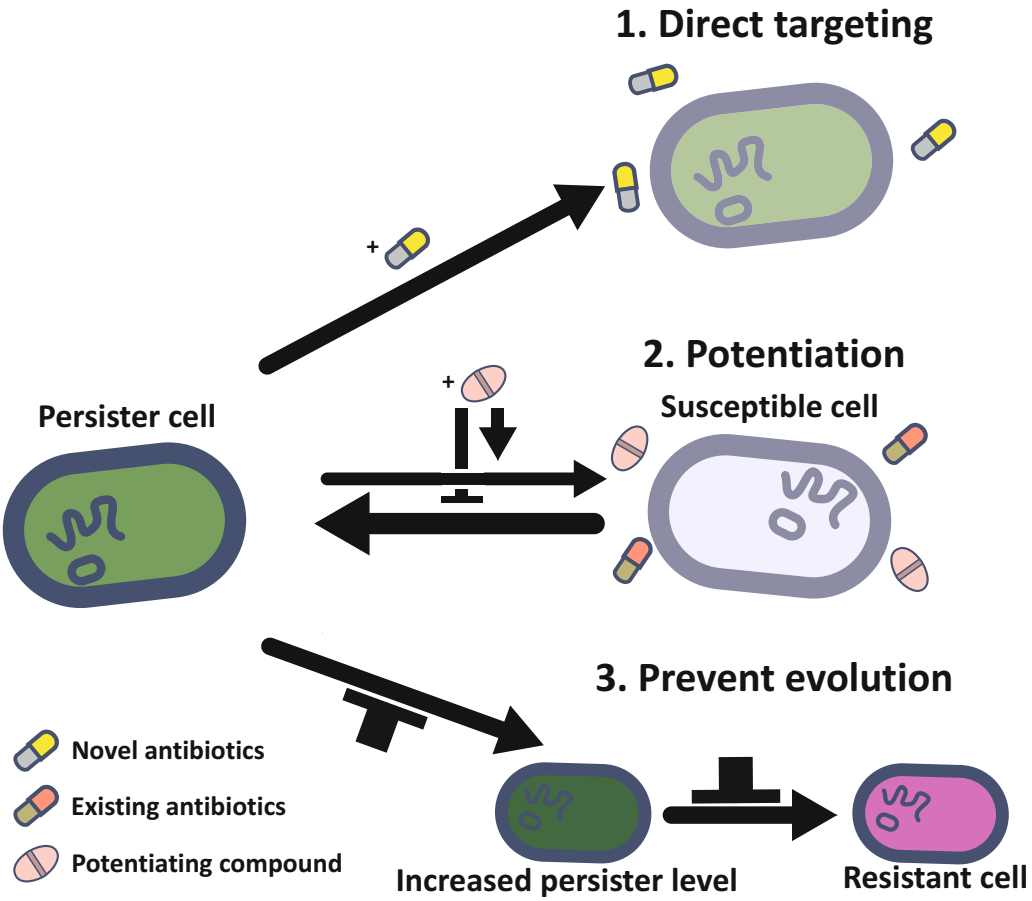

Figure 4. Strategies to combat persistence.
(1) Direct targeting of persister cells using single-drug therapy with novel antibiotics or combinatorial therapy with existing antibiotics. (2) Combining existing antibiotics with a potentiating compound to enhance the efficacy of the antibiotic against the whole population, including both persister and nonpersister cells. Potentiating compounds can inhibit persister formation, trigger persister recovery and regrowth, and increase antibiotic uptake or decrease antibiotic efflux. (3) Preventing evolution of increased levels of persisters, which may also limit the evolution and spread of resistance via persistence.

Another option to decrease the number of persisters is the use of nonantibiotic potentiating compounds as adjuvants to existing antibiotics, which can improve the efficacy of the antibiotics against a population of both sensitive and persister cells (Fig 4). One possibility to increase the killing of both sensitive and persister cells is to increase the intracellular antibiotic concentration. Antibody–antibiotic conjugates, where an antipathogen antibody is linked to an antibiotic, increase the specificity of their delivery, while minimizing potential toxic side effects (Lehar *et al*, 2015; Zhou *et al*, 2016; Mariathasan & Tan, 2017). Moreover, an increased intracellular antibiotic concentration can be achieved by increasing the uptake of the antibiotic with compounds that cause direct membrane permeabilization such as colistin (Cui *et al*, 2016; Chung & Ko, 2019) or substances that activate mechanosensitive ion channels (Jiafeng *et al*, 2015; Chen *et al*, 2019; Lv *et al*, 2022). Additionally, efflux pump inhibitors can also increase intracellular antibiotic concentrations (Adams *et al*, 2011; Pu *et al*, 2016). The effectiveness of an antibiotic therapy on a population of bacteria may also be improved by decreasing the number of antibiotic-tolerant persister cells. One strategy to lower persister levels is by preventing their formation with quorum sensing inhibitors (Starkey *et al*, 2014; Allegretta *et al*, 2017), antioxidants (Rowe *et al*, 2020) or by inhibiting toxin–antitoxin systems using toxin inhibitors (Li *et al*, 2016). However, a notable downside of using antioxidants is that this could result in concomitantly lower beneficial ROS-mediated mechanisms of the immune system (Dumas & Knaus, 2021), leading to increased infections and longer infection durations. Preventing active persistence mechanisms by decreasing active stress responses such as the SOS response using RecA inhibitors (Alam *et al*, 2016; Yamamoto *et al*, 2022), the general stress response using mesalamine (Dahl *et al*, 2017) or the stringent response by limiting ppGpp production (Nguyen *et al*, 2011; Dutta *et al*, 2019) has also been associated with lower persister levels and an improved treatment in animal infection models. Another strategy to decrease the number of persisters in a population is by resensitizing them to antibiotics by inducing their regrowth. Persister regrowth can be stimulated by administering signaling molecules like indoles (Song & Wood, 2020b), although the effects are strain and antibiotic specific (Sun *et al*, 2020). Moreover, many carbon sources are known to restart the metabolism and thereby either increase the PMF and the uptake of the antibiotic in

the cell (Allison *et al*, 2011; Kitzenberg *et al*, 2022) or induce persister regrowth (Allison *et al*, 2011; Vilchèze *et al*, 2017).

Another strategy, parallel to the ones mentioned earlier, could be to restrict or reverse the evolution of antibiotic persistence (Fig 4). Combination therapy or antibiotic cycling has been suggested as potential approaches to prevent the evolution of resistance against commonly used antibiotics (Michel *et al*, 2008; Baym *et al*, 2016). These strategies rely on negative evolutionary interactions between antibiotics, known as collateral resistance, where a mutation that confers resistance to one drug concomitantly decreases resistance to another. Conversely, resistance to one drug leading to resistance to another is referred to as cross-resistance. However, it is typically observed that the evolution of increased persistence to a specific drug is accompanied by increased persistence to other drugs, including those of other classes, thus limiting the potential use of mixing or cycling antibiotic (Michiels *et al*, 2016; Van den Bergh *et al*, 2016; Lázár *et al*, 2022). Nonetheless, the patterns of cross-persistence and collateral persistence interactions remain poorly understood and further research may identify promising antibiotic combinations. Additional methods, such as blocking evolvability factors, may also be promising to prevent persistence evolution. For instance, targeting mutagenesis-promoting factors, such as Mfd, the mycobacterial mutasome or SOS response regulators, could be a potential approach to prevent the evolution of persistence (Ragheb *et al*, 2019; Merrikh & Kohli, 2020). By reducing the number of persisters or limiting high-persistence evolution, the proposed strategies can not only combat antibiotic persistence and their role in persistent infections but may also slow down the evolution of antibiotic resistance. This could prolong the effectiveness of existing antibiotics and help to preserve the usefulness of these critical drugs in the future, which is particularly important given the decreasing rate of discovery of novel antibiotics.

Concluding remarks

Bacterial pathogens are notorious for their remarkable adaptability in hostile environments, including their ability to persist in the presence of otherwise lethal doses of antibiotics. In this review, we have outlined recent advances in our understanding of antibiotic persistence. Current research focused on persister formation, recovery, and regrowth has reinforced the notion that persistence is a complex multifaceted and adaptive phenomenon that is significantly influenced by environmental cues. This highlights the importance of considering the ecological context in which bacteria exist, including intra- and interspecies communication and interactions with the immune system. Acknowledging this wider ecological framework is essential for a better understanding of antibiotic persistence as an emergent property of the microbial community in its natural environment, rather than solely determined by the characteristics of individual bacterial cells. This will require researchers moving beyond conventional methods that rely on homogeneous isogenic cultures and instead develop techniques that can capture the heterogeneity and complexity of bacterial populations *in vivo*. However, there exists a relative paucity of antibiotic persistence research beyond well-mixed laboratory conditions. Therefore, it is critical to contextualize basic research findings in *in vivo* mammalian models or clinical settings (Box 1). Recent research conducted with murine models appears to hold great potential for translating *in vitro* findings (Newson *et al*, 2022; preprint: Verstraete *et al*, 2022a). Additionally, several promising strategies have been proposed to combat antibiotic persistence, but their efficacy is yet to be validated. As a first step toward this validation, further development and utilization of murine models may prove to be valuable. In conclusion, further research is necessary to obtain a more holistic understanding of bacterial persistence and its implications in clinical settings, potentially leading to the development of effective therapies for persistent bacterial infections.

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The authors declare that they have no conflict of interest.

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