# Evolutionary causes and consequences of bacterial antibiotic persistence

*Erik Bakkeren*[1], *Médéric Diard*[2]✉ *and Wolf-Dietrich Hardt*[1]✉

Abstract | Antibiotic treatment failure is of growing concern. Genetically encoded resistance is key in driving this process. However, there is increasing evidence that bacterial antibiotic persistence, a non-genetically encoded and reversible loss of antibiotic susceptibility, contributes to treatment failure and emergence of resistant strains as well. In this Review, we discuss the evolutionary forces that may drive the selection for antibiotic persistence. We review how some aspects of antibiotic persistence have been directly selected for whereas others result from indirect selection in disparate ecological contexts. We then discuss the consequences of antibiotic persistence on pathogen evolution. Persisters can facilitate the evolution of antibiotic resistance and virulence. Finally, we propose practical means to prevent persister formation and how this may help to slow down the evolution of virulence and resistance in pathogens.

Antibiotic
An antimicrobial agent that either inhibits (bacteriostatic antibiotic) or kills (bactericidal antibiotic) bacteria.

Resistance
The genetically encoded ability of cells to grow in the presence of an antibiotic. Resistance increases the minimum inhibitory concentration of an antibiotic compared with susceptible cells. The offspring remains resistant, even if grown in the absence of antibiotics.

[1]*Institute of Microbiology, Department of Biology, ETH Zurich, Zurich, Switzerland.*
[2]*Biozentrum, University of Basel, Basel, Switzerland.*
✉*e-mail: mederic.diard@unibas.ch; hardt@micro.biol.ethz.ch*

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Antibiotic treatment failure is a substantial problem in modern medicine. Although this has been largely attributed to the emergence and spread of antibiotic resistance in pathogenic bacteria[1], mechanisms other than resistance can also reduce the susceptibility of microorganisms to antimicrobials. Within a few years of penicillin use in clinics, it became clear that antibiotic treatment often failed to completely eliminate populations of susceptible bacteria, even if they lack genetic resistance determinants[2,3]; some cells of the bacterial population, termed 'antibiotic persisters' (persisters in short), were able to survive treatment. These observations led to an avenue of research aiming to understand the mechanisms behind bacterial persistence and to develop strategies to combat these recalcitrant bacteria. However, the link between bacterial persistence and pathogen evolution is understudied. In particular, persistence can lead to the evolution of antibiotic resistance and virulence. In this Review, we collate evidence regarding the selective forces that promote persistence and discuss its evolutionary consequences.

## Definitions and mechanisms

Bacteria can survive antibiotic treatment owing to four different phenomena: resistance, heteroresistance, tolerance and persistence[4–6]. Of these, mechanisms leading to resistance remain the most studied and best understood[7–9]. Resistance to antibiotics is generally determined genetically and typically protects the strain against a particular class or group of related antibiotics. Genetic resistance can occur through mutation in the bacterial chromosome (that is, mutational resistance, reduced drug binding to target, increased efflux pump expression and so forth) or through the acquisition of bona fide resistance genes through horizontal gene transfer (HGT)[10]. These transferred resistance genes often encode detoxifying enzymes or additional efflux pumps[10]. In either case, the genetic change raises the minimum inhibitory concentration (MIC) of the drug required to inhibit growth or kill bacteria (thus, resistant bacteria have an increased MIC)[4,11]. In most cases, resistance pertains to all cells of the resistant population. In some cases, the resistance phenotype is only expressed by some cells of a clonal bacterial population (that is, heteroresistance). Traditional MIC assays can miss heteroresistance, as resistant subpopulations can quickly outcompete the susceptible cells if antibiotics are applied[5,11–15]. Tolerance is the ability of genetically susceptible bacteria to survive concentrations of a bactericidal antibiotic above the MIC. Contrary to resistance, tolerant bacteria cannot replicate in the presence of the antibiotic but are simply killed at slower rates[4,16,17]. Antibiotic persistence is similar to tolerance, in that the MIC of these bacteria does not change; however, the main difference is that antibiotic persistence only affects a subpopulation of bacteria exposed to the antibiotic (this means that persistence and tolerance are the same if 100% of the population is persistent)[4]. Thus, antibiotic persistence could be referred to as heterotolerance. Therefore, in the presence of bactericidal antibiotics, killing curves for bacteria engaging in persistence will be biphasic. Sensitive bacteria will be killed quickly, leading to a fast decrease of the surviving population, in parallel with a slow decrease of the persister subpopulation that is only revealed in the second part of the killing curve[4,6,18] (FIG. 1).

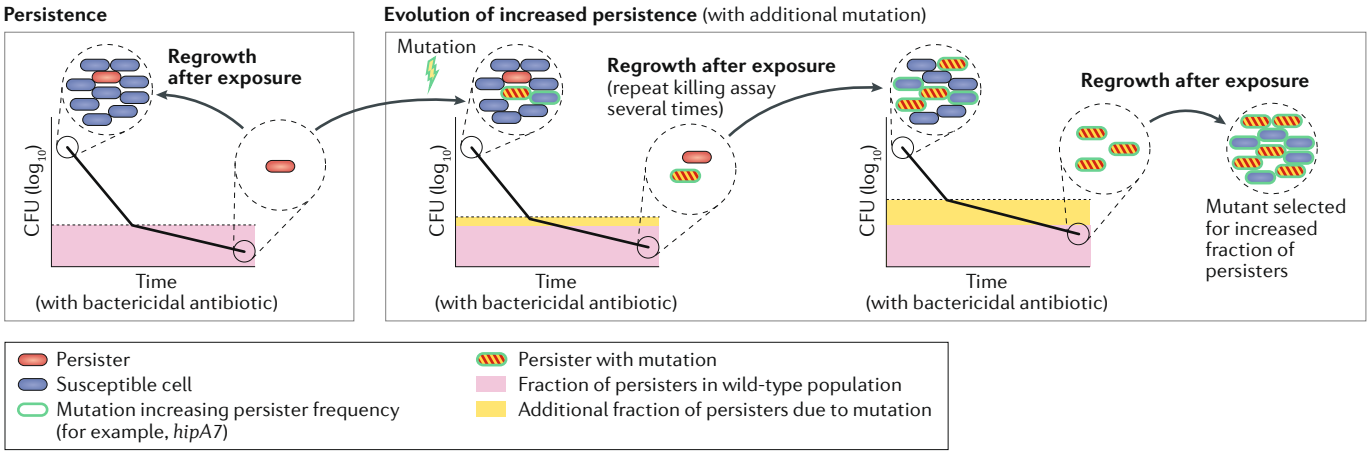


Fig. 1 | **Evolution of higher persistence.** Persisters are observed in simple killing assays in which a culture of bacteria is treated with a bactericidal antibiotic. Biphasic killing occurs, in which the susceptible subpopulation (blue) is killed quickly in parallel with much slower kinetics of killing for persisters (red), which is revealed after the susceptible subpopulation is eliminated. Regrowth of the survivors without antibiotics, followed by another antibiotic treatment, leads to an identical biphasic killing pattern (left panel). In some cases, mutations can occur (such as in the *hipA7* high-persistence mutant, indicated with green outline; yellow diagonal stripes show persisters of that genotype) that increase either the fraction of persisters (as depicted in the right panel) or the rate at which the persisters are killed (flattened persister killing curve; not shown). These mutations are heritable and will increase the number of survivors in the presence of antibiotics. Red shading indicates the populations of persisters in a wild-type strain. Yellow shading indicates the increase in fraction of persisters after evolution and selection for a high persister mutation. CFU, colony-forming unit.

Which molecular mechanisms lead to antibiotic persistence (or tolerance) in a bacterial cell? Persistence is generally a non-inherited phenotype, which is observed in all studied bacterial species. Populations of genetically identical bacteria tend to form smaller or larger subpopulations that transiently show tolerance. Spontaneous persistence refers to situations in which the size of the tolerant subpopulation is independent of the tested environmental cues. However, in many situations, the size of the tolerant subpopulation is regulated in response to environmental stimuli. This phenomenon is termed triggered persistence[4]. The molecular basis of persister formation is a matter of debate and seems to involve slow growth[19], the cellular ATP pool, the proton gradient and/or blockage of protein biosynthesis (reviewed elsewhere[6,11,18]). Additionally, mutations in tRNA synthetases, essential enzymes involved in protein biosynthesis, or toxin–antitoxin systems can increase tolerance or persistence[20,21]. For example, the *hipA7* mutation (*hipA* encodes a toxin) is well characterized in *Escherichia coli* and increases the fraction of persisters in a population by around 100-fold[22,23]. Thus, different mechanisms may promote persister formation in response to particular cues.

Based on the idea that persistence can increase the fitness of a particular clone depending on environmental conditions, the factors that govern its evolution are genetically encoded and should therefore be selectable and heritable (FIG. 1). Mutants featuring increased tolerance (that is, less steep killing curves in the presence of a bactericidal antibiotic) or increased fractions of antibiotic persistent cells can be identified and selected for during experimental evolution[20,21,23–28]. Thus, higher persistence can indeed evolve. The relevant clinical settings and the selection pressures driving this evolution are discussed in the next paragraphs.

## Persistence in the clinic

There are important differences between clinical persistence (for example, from a long-lasting, persistent infection that the host fails to clear) and antibiotic persistence. These definitions sound similar, particularly in modern times when antibiotics are often used to treat persistent infections. However, although the underlying mechanisms may have equivalent roots in some cases, they differ in others. Numerous pathogens, such as *Mycobacterium tuberculosis*, *Salmonella enterica*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *E. coli*, engage in persistent infections (reviewed elsewhere[29]). Often, the root cause is ineffective clearance by the host as a result of immune deficiency, subversion or evasion[29–31]. When treated with antibiotics, substantial subpopulations of these pathogens survive in the tissue. However, whether these survivors are truly persisters or not is often unclear. This uncertainty stems from difficulties distinguishing survival driven by antibiotic persistence from a 'trivial' lack of penetration by the antibiotic used[32] or the induction of resistance genes in vivo. Nevertheless, there are several studies that have shown the role of antibiotic persistence during infection (reviewed elsewhere[6]; specific examples below).

As discussed in more detail below, it is tempting to speculate that antibiotic persistence may coincidentally evolve during persistent infection, at least in some cases. Persistent infection evolves by promoting the transmission of the pathogen in host populations, regardless of antibiotic use[33]. However, the standoff between the immune response of the host and virulence factors of the

**Persisters**
Cells belonging to a subpopulation that is killed much slower than the rest of the population during exposure to bactericidal antibiotics. Typically, persisters halt growth during this survival. However, they can re-engage in fast growth when the antibiotic is removed.

**Persistence**
The phenomenon that for a population in which two or more distinct subpopulations exist (susceptible and tolerant), treatment with a bactericidal antibiotic will kill the susceptible subpopulation quickly, simultaneous with a much slower killing of the tolerant subpopulation. This leads to biphasic killing curves characteristic of persistence. Persistence is not heritable (clones isolated from the tolerant subpopulation will again give rise to a mix of susceptible and tolerant cells). Persistence can also be called heterotolerance.

Heteroresistance
The ability to grow a subpopulation of cells in the presence of an antibiotic. This subpopulation can be the result of rare resistant mutants that increase in frequency over time (polyclonal heteroresistance) or two distinct subpopulations (sensitive and resistant) that switch back and forth phenotypically even in the absence of antibiotics. In the latter case, the antibiotic exerts a selective pressure that can change the relative frequency of sensitive versus resistant cells (monoclonal heteroresistance). In standard minimum inhibitory concentration (MIC) assays, this increases the MIC of an antibiotic compared with a population of susceptible cells (when the inoculum is grown without antibiotics).

Tolerance
The ability of cells to survive in the presence of a bactericidal antibiotic to a higher extent than susceptible cells. This phenomenon pertains to all cells of the population and increases the minimum duration of killing in the presence of an antibiotic.

Horizontal gene transfer
(HGT). The transfer of genetic information from one organism to another. In bacteria, the main mechanisms are conjugation (mediated by plasmids), transduction (mediated by phages) or transformation (uptake of DNA from the environment).

Spontaneous persistence
Persistence observed without any stimulus; subpopulations of tolerant cells exist even during growth when environmental parameters are kept optimal.

Triggered persistence
Persistence that arises in response to a certain stimulus. This stimulus can result from stressful conditions in which it can be beneficial to maintain minimal metabolic activity in a subpopulation of cells.

pathogen during persistent infection can coincidentally yield tolerant subpopulations (by triggered or spontaneous persistence). However, as this is difficult to test in the clinical context, animal models may offer a unique opportunity to verify this hypothesis.

For example, long-term shedding associated with persistent infection of *S. enterica* subsp. *enterica* serovars such as Typhi (*S.* Typhi) and Typhimurium (*S.* Typhimurium) can occur in humans and animals[34]. Persistent infection has been recapitulated in mouse models for *S.* Typhimurium, showing that shedding can occur for extended periods of time[35,36]. Recently, *S.* Typhimurium cells associated with persistent infection were found to reside in granulomas of alternatively activated (M2-like) macrophages in the spleen[37]. This is in line with in vitro evidence suggesting that *S.* Typhimurium polarizes macrophages towards an M2-like phenotype to enable better survival within cells, including during antibiotic therapy[38]. Indeed, the *S.* Typhimurium–macrophage interaction generates substantial heterogeneity in both bacterial and host cell populations, which can influence survival during antibiotic therapy[38–41]. This finding is recapitulated in mouse models in which *S.* Typhimurium persisters have been studied[42–45].

Additionally, *S.* Typhi forms gallstone-associated biofilms, which lead to a persistent carrier state and increased transmission over long periods of time[46]. Concurrently, large fractions of the biofilm-lodged bacteria will survive antibiotic treatment, despite lacking genetic resistance. However, this may be attributable to either the lack of antibiotic penetration of the biofilm or true persistence.

Cystic fibrosis leads to persistent lung infection and is often associated with biofilms that contain heterogeneous populations of *P. aeruginosa*. These associations are mostly correlative, as definitive in vivo evidence that biofilms form during cystic fibrosis is insufficient[47]. This uncertainty can likely be explained by the lack of adequate standardized animal models to recapitulate clinical observations, although recently some advances have been made[47,48]. In any case, biofilms include large subpopulations of bacterial cells that will survive treatment with antibiotics even in the absence of corresponding genetic resistance determinants. Rates of persisters have been cited to be up to 1,000-fold higher in biofilms compared with growing planktonic cultures in vitro[49]. This is possibly related to the plethora of signalling molecules and stress signals produced within and exchanged between bacteria in biofilms, such as (p)ppGpp in the stringent response and mediators of the SOS response[18,49]. Indeed, transcriptional profiling of *P. aeruginosa* from lungs of people with cystic fibrosis has shown upregulation of stress response genes (oxidative, osmotic and antibiotic stress, SOS response and mediators of the stringent response)[50–52]. Therefore, recalcitrance in biofilms could involve antibiotic persistence, but we cannot exclude poor antibiotic penetration into the biofilm. Regardless, an increasing number of studies have linked the persistent carriage of *P. aeruginosa* to evolutionary changes, such as a tendency for decreased virulence or increased antibiotic resistance and persistence[53–56]. Clinical administration of intermittent doses of antibiotics to people with cystic fibrosis revealed transient reductions of *P. aeruginosa* in sputum samples, but these reductions became less pronounced over time[55]. The lack of resistance in isolates from these patients indicates an evolution towards tolerance or persistence[55]. In agreement with this finding, high-persistence mutants have been isolated from patients with cystic fibrosis[56].

*S. aureus* also engages in persistent, relapsing infections that are often difficult to clear with antibiotics, such as osteomyelitis[57,58]. Poor penetration of antibiotics into bone or biofilms of *S. aureus* may be a driving factor. However, *S. aureus* also forms small-colony variants, which resist host defences, evade immune activation and are often associated with clinical persistence of *S. aureus*[57]. More recently, some small-colony variants were shown to result from a long lag time before resuming growth after isolation from patient or mouse abscesses, or after growth under other stresses, such as low pH[59]. These small-colony variants were shown to be antibiotic tolerant[59], suggesting a correlation between persistent infection in a host and antibiotic persistence.

Similarly, *M. tuberculosis* establishes persistent infections that are difficult to treat with antibiotics[60]. In patients with *M. tuberculosis* who undergo antibiotic therapy, several subpopulations with unique resistance profiles evolve during the course of an infection[61]. Additionally, in animal models, resistance-independent mycobacterial survival during antibiotic therapy has been demonstrated[62,63]. Increased antibiotic persistence has been shown to result from stress-induced noise in RNA expression based on nutrient limitation and subpopulations of growing and non-growing but metabolically active bacteria[64,65]. Such heterogeneity is suggested to be the result of asymmetrical cell division[66], at least in ex vivo experiments.

Numerous additional examples exist supporting a link between persistent infection and antimicrobial persistence. These observations may extend beyond the bacterial kingdom. Fungal pathogens such as *Candida* spp.[67,68] and viruses that can integrate into host genomes, such as HIV and herpesviruses[69,70], show similar patterns of coincidental evolution of clinical persistence and resistance-independent recalcitrance to antimicrobial therapy.

## Why does persistence emerge?

As discussed above, all tested bacterial populations always include a small fraction of persisters that are attributable to spontaneous persistence. However, why can bacteria evolve towards forming bigger subpopulations of persisters or slower killing rates? Tolerance and persistence enable survival in different harmful conditions (for example, survival in the presence of diverse classes of antibiotics)[4,71]. If exposure to antibiotics selects for persistence and tolerance, these traits likely emerged originally to promote survival in stressful environments in which antibiotics play a small role, although naturally produced antibiotics that mediate interspecies competitions may have contributed in some cases[72] (for example, nutrient limitation, stressful compounds from the environment and phage–host dynamics)[73,74]. Therefore,

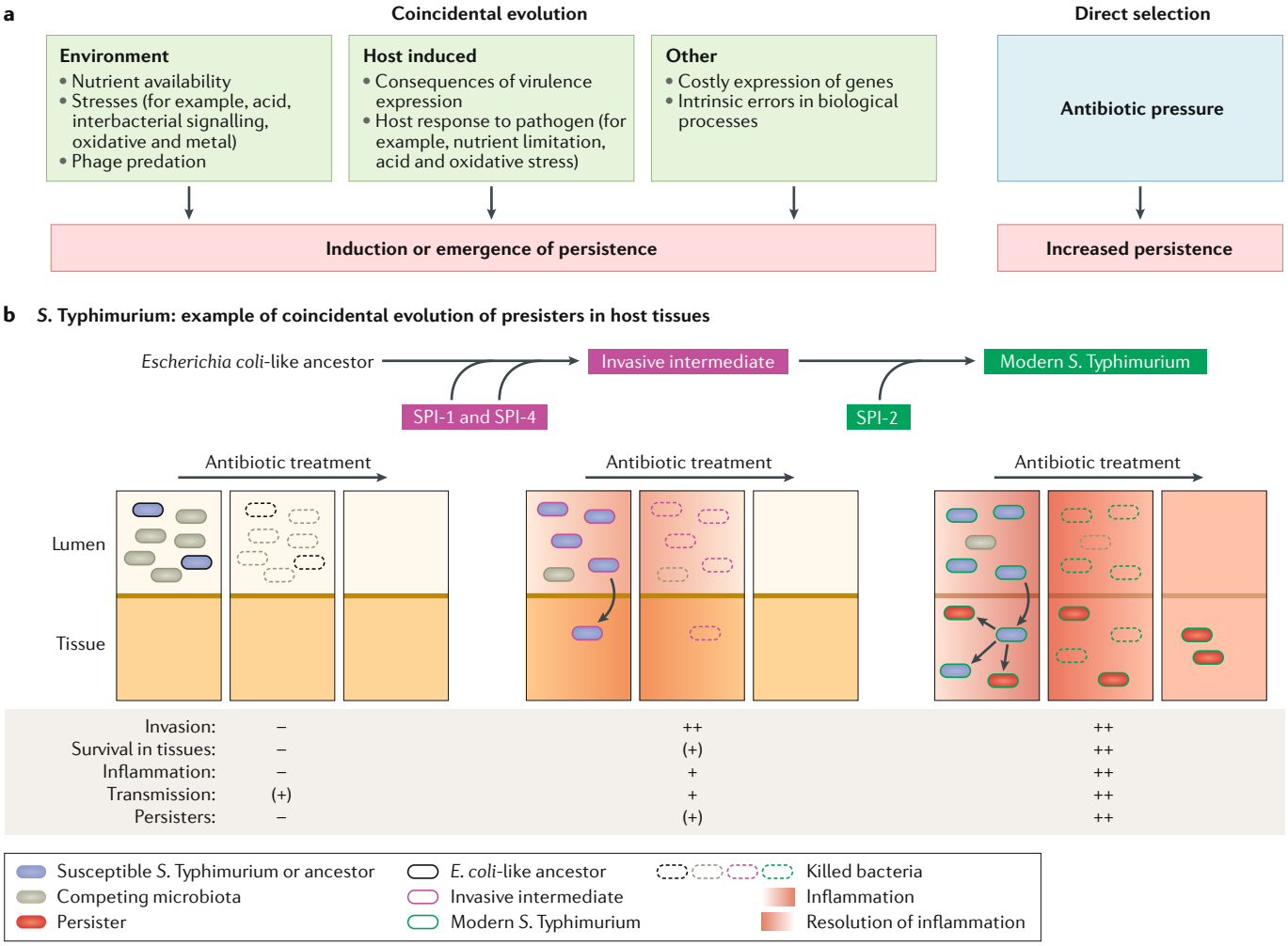


Fig. 2 | **Selection for persistence. a** | Responses to environmental and intrinsic stresses favour the formation of subpopulations of cells tolerant to antibiotics, namely persisters. These include conditions in the host and burdens associated with virulence expression. Repeated exposure to antibiotics can select for mutations that increase the fraction of persister cells. **b** | An example of persistence was coincidentally selected with the pathogenic lifestyle of *Salmonella enterica* subsp. *enterica* Typhimurium. The acquisition of virulence factors coincides with persistence in this pathogen. This effect is due to its ability to invade (acquisition of *Salmonella* pathogenicity island 1 (SPI-1) and SPI-4) and to survive and grow in host tissues over long periods of time (acquisition of SPI-2 and other virulence factors)[91,92]. Virulence factors enable S. Typhimurium to trigger inflammation and boost its transmission, but indirectly lead to persistence in tissue reservoirs. Invasion and gut colonization are shown on the left at each stage of S. Typhimurium evolution in the absence of antibiotics; the consequence of the evolution of virulence on survival during antibiotic treatment is shown in the middle and on the right. Susceptible S. Typhimurium cells are indicated in blue, persisters in red and competing microbiota in grey. Killed bacteria are shown as dotted, hollow rods. Inflammation is depicted as shades of red.

Clinical persistence
The failure of either the immune system or antimicrobial therapy to eliminate the pathogen, resulting in the pathogen remaining in the host for long periods of time. That is, clinical persistence can be the result of either antibiotic persistence or persistent infection (for example, as a result of impaired immunity, immune subversion or evasion, biofilm formation or intracellular survival).

diverse factors may coincidentally have driven the evolution of bacteria that can form increased fractions of persistent cells or feature tolerance (FIG. 2). The speculative aspect of this reasoning reflects our inability to reliably predict how genotype changes affect persistence phenotypes and also the lack of a comprehensive understanding of all relevant evolutionary forces and their relative contribution to the evolution towards emergence and increased fractions of persistent cells.

***Antibiotic selection for increased persistence.*** Our societal concern with the success or failure of antibiotic therapies has focused our thinking on persistence as a means for bacteria to survive antibiotic exposure. Clearly, persistence-mediated failure of an antibiotic therapy itself is worrisome. However, can antibiotic exposure select for mutants with reduced killing rates or that can form larger persistent subpopulations?

Indeed, intermittent exposure to antibiotics in cultures in vitro can select for the evolution of clones with increased tolerance or larger fractions of persisters than the original clone under the selection regime applied[20,21,23–28]. As mutations increasing persistence are rarer than those increasing tolerance[20], they may be overlooked as tolerance mutations affect the entire population and would mask mutations that affect subpopulations. Similarly, during persistent infections associated with long-term antibiotic treatments, strains of

Persistent infection
The pathogen is not cleared from the host but remains in specific cells or compartments of the host for long periods of time, independently of antimicrobial treatment. Persistent infection can lead to clinical persistence.

Biofilms
A collection of microorganisms that adhere to each other and surfaces, embedded within an extracellular matrix. Exchange of nutrients, chemical messengers and genetic information is prominent, promoting a heterogeneous mixture of cells, including dormant cells. Biofilms are typically recalcitrant to antibiotic therapy (through poor antibiotic penetration, antibiotic persistence or both).

Stringent response
A stress response in bacteria as a result of nutrient limitation or other stress conditions that is mediated by accumulation of the alarmone (p)ppGpp. (p)ppGpp influences the transcriptional profile of the cell, for example to favour general metabolism maintenance rather than ribosome biosynthesis.

SOS response
A response to damage-inducing stresses detected by single-stranded breaks in DNA stalling the DNA polymerase. This induces LexA-repressed genes, which often include error-prone DNA repair and inhibitors of cell division.

Bet-hedging
An evolutionary strategy in which part of the population has decreased fitness in favourable conditions but is able to survive after a shift to more stressful environments. In bacteria, bet-hedging can occur when more than one phenotype is expressed at a population scale. One phenotype promotes optimal growth in the present environment, whereas others grow or survive suboptimally in this environment but would be more fit if the conditions changed. This mixture of phenotypes leads to an optimal fitness of the entire population over time under changing conditions.

pathogens such as *P. aeruginosa*, *E. coli* or *Staphylococcus* spp. have been isolated with high levels of persistence; that is, slower complete killing by bactericidal antibiotics and biphasic killing curves are observed[56,75–78]. This can be the result of a larger subpopulation that engages in persistence. Alternatively, the persister fraction remains the same but dies slower than before selection. This process makes sense if one considers spontaneous persistence as a bet-hedging strategy to overcome episodic stress, including antibiotic treatment. Thus, the evolution driven by direct selection can increase the size (or decelerate the killing kinetics) of the surviving subpopulation when an antibiotic is encountered.

***Induction of persistence by other stresses: an example of coincidental evolution.*** As mentioned, bacteria must overcome diverse stressful environments[79]. Many survival strategies also increase antibiotic persistence. In these cases, the selection for persistence is likely based on ancestral survival needs in bacteria rather than the contemporary use of antibiotics in medicine and farming practices; it is unlikely that antibiotic pressure has had a major role in the existence or the original emergence of persistence. Changes in nutrient abundance or composition clearly influence microbial growth dynamics. The best-characterized trigger of persistence is nutrient starvation[22]. Bacteria in the stationary phase often exhibit much larger subpopulations of persisters than those in the exponential phase, and the addition of glucose or oxygenation sensitizes bacteria to antibiotic treatment[80]. Antibiotic persistence has also been observed in subpopulations after changes in the carbon source; in this case, one subpopulation grows on the new carbon source whereas the other subpopulation remains dormant (not metabolically active) and therefore recalcitrant to antibiotics[81]. These examples generate responsive diversification, in which more than one phenotype is generated after a stimulus[81]. Mechanistic understanding of triggered persistence revealed a role for stress responses, particularly for the second messenger and 'alarmone' (p)ppGpp, which actively regulates the switch from growth to metabolic homeostasis and survival[82,83]. Thus, it is plausible that the balance of metabolic needs for growth versus survival by dormancy should select for antibiotic persistence.

Besides nutrient availability, other stresses can also induce the formation of persisters. This includes acid stress[74], interbacterial signalling at high cell densities[84–86], oxidative stress[74,87] and toxic concentrations of metals[88]. A link between phage predation of bacteria and persistence has also been established[89]. In this case, induction of prophages was reduced in persisters (but not their susceptibility to infection by phages). This observation suggests that both phages and their host bacteria may benefit from persistence in stressful environments, which limits cell lysis by prophage induction to fast-growing bacterial cells. As mentioned by Gefen and Balaban, this result is of interest not only for studying bacteria–phage interactions but also for explaining conditions in which predation by temperate phages may have shaped the evolution of persistence[73,89]. Contrary to the evolution of persistence by antibiotic selective pressure, it is unclear whether the stresses that can induce persistence function to further evolve higher levels of persistence. However, collectively they provide a tempting explanation for the existence or emergence of persistence. Little work has been done to address whether these stresses can actually evolve higher antibiotic persistence experimentally.

There are several mechanisms by which these stresses could contribute to induce or evolve persistence. The increase in the lag time that cells take to resume growth after a stress (for example, during exit from the stationary phase) can increase persistence by enlarging the size of the persistent subpopulation[21,22]. In addition to lag time as a result of general slow growth, it seems that persister cells themselves have a specific responsiveness to different antibiotics depending on their target within the cell and the metabolic activity of that particular cellular target[90]. For example, the fraction of *E. coli* surviving treatment with the gyrase inhibitor ciprofloxacin was much smaller in mutants lacking active double-strand break repair than in wild-type *E. coli*. By contrast, no such difference was observed in the survival of antibiotics that target other cellular functions, such as ampicillin and gentamycin[90]. Therefore, individual cells may be recalcitrant to different antibiotics, but collectively they contribute to multidrug persistence or tolerance. Altogether, various stresses can represent a selective pressure to increase persistent subpopulations, decelerate their death after antibiotic exposure, prolong the lag time and/or reduce the metabolic activity of specific cellular processes (FIG. 2a).

***Indirect selection for persistence.*** Thus far, we have described selective forces directly leading to the evolution of persister subpopulations that are larger (or killed slower) than expected from spontaneous persistence. This process can occur through either the use of antibiotics or the presence of natural stressors, during which a general survival strategy such as persistence (which impacts antibiotic susceptibility) is advantageous. However, there are also less intuitive factors that may lead to increased persistence (FIG. 2).

A prominent example is the evolution of virulence factors that promote the intracellular lifestyle of pathogens. As discussed above, stresses from pathogen–host interaction may provide a selection for persistence (to avoid or subvert the immune response). In the case of *S.* Typhimurium, the evolution of virulence likely led to a selection for increased survival during antibiotic therapy (FIG. 2b). *S.* Typhimurium encodes two main pathogenicity islands: *Salmonella* pathogenicity island 1 (SPI-1) and SPI-2. These islands affect the pathogen–host interaction in two different ways[33,91,92]. They enable the pathogen to elicit mucosal inflammation to boost gut-luminal blooms and accelerate transmission[42,93], but also enable *Salmonella* spp. to survive and grow intracellularly, which indirectly facilitates the formation of antibiotic persisters[40]. Additionally, it was recently shown that a host factor, SLC11a1, which restricts systemic growth of pathogens such as *M. tuberculosis* and *S.* Typhimurium by depleting local $Mg^{2+}$ availability, generates heterogeneous bacterial growth rates associated with heterogeneous gene expression patterns[94]. This finding indicates

Nutrient starvation
A cell is faced with no or insufficient nutrients to grow and must therefore use its own reserves, or rely on dormancy to survive.

Dormant
A state of reduced metabolic activity and halted growth that can protect bacterial cells against antibiotics that target aspects of cellular growth or metabolism. Dormancy is a mechanism by which cells are tolerant or persistent.

Responsive diversification
The generation of a range of different responses to a certain stimulus. In bacteria, for example, several subpopulations expressing different phenotypes can emerge in response to stressful conditions, favouring survival in changing environments.

that the host itself may also induce persistence in the infecting pathogen population. This indirect evolution of persistence likely explains the recalcitrance of *S.* Typhimurium in mouse models during antibiotic therapy[42–45,95]. Moreover, virulence factor expression carries a fitness cost[96], and the reduced growth rate can increase persistence, at least in vitro[97]. Further work will be needed to show how this in vitro phenotype may translate to in vivo infections. Nonetheless, virulence factors in other intracellular pathogens that show persistence, such as *M. tuberculosis*, *E. coli*, *Shigella* spp., *Yersinia* spp. and *Listeria* spp.[11], may lead to a similar indirect selection for antibiotic persistence.

Indeed, in the context of an infected host under antibiotic treatment, it is tempting to consider persistence as an additional virulence factor and the antibiotic as a supplement to the antimicrobial defence cocktail elicited by the host immune defence. In a similar manner to the arms race between pathogen virulence factors and host immune responses[98], persistence and antibiotic treatment are intimately linked. Akin to antibiotic treatment, the goal of the host immune response is to limit pathogen population sizes. For example, in the case of *S.* Typhimurium, phagocyte-intrinsic restriction mechanisms, including NADPH oxidase, antimicrobial peptides and nutrient depletion inside the phagosomes of phagocytic cells, restrict pathogen population sizes at systemic sites[99]. Correspondingly, *S.* Typhimurium uses defensive virulence factors, such as superoxide dismutases and SPI-2, to mitigate host cellular responses and thereby combat these defences[94,99–104]. Thus, the evolution of SPI-2 has improved survival and growth within a host. Strikingly, most infected cells in a host contain only one or very few bacteria at once, and the intracellular growth rate of the pathogen is very slow[40,43,45]. Thus, improved host colonization also leads to higher loads of slowly growing bacteria in host organs. Importantly, SPI-2 has also been implicated in persistence to antibiotics[38,44]. This provides a first example illustrating why stresses from the immune response not only may have selected for persistence directly but may have selected for the evolution of compensatory virulence factors and by extension for elevated levels of persistence.

As slow growth and/or metabolic dormancy are often associated with persistence, it is logical that metabolically costly traits in bacteria lead to increased persistence. In fact, this is often the case for toxin–antitoxin systems, in which the toxin component limits the metabolic potential of the bacteria (which is only rectified by the cognate antitoxin)[105]. However, as is the case with SPI-1-mediated virulence in *S.* Typhimurium[96,97], such costly systems clearly have fitness advantages for the bacteria that extend beyond simple survival in changing environments. Therefore, in the case of pathogens causing invasive, persistent infections, one can speculate that the evolution of costly systems, such as virulence factors, antibacterial competition factors or essential niche-dependent biosynthetic pathways, may lead to indirect selection for increased persistence. An example of this was elegantly shown through ectopically expressing costly non-toxin proteins in *E. coli*[106]. This expression increased the population size that survived antibiotic treatment. This effect could be a result of increased tolerance if overexpression occurred equally in each cell, or of persistence if costly protein expression occurred unevenly within the population. Given this finding, the authors speculated that stochastic variation in gene expression for costly proteins may affect persistence (and also potentially explain spontaneous persistence[106]).

Altogether, there are several examples of coincidental evolution of phenotypes that lead to increased persistence. In these examples, the selective force does not act on persistence itself but, rather, enables bacteria to survive or exist in niches or engage in phenotypes that can either induce or enable persistence.

## Evolutionary consequences

Antibiotic persistence has some very important evolutionary consequences. In addition to the obvious clinical consequences of persistence for the infected host, it is a facilitator for the evolution of other traits. This includes antibiotic resistance itself and virulence (FIG. 3).

***Antibiotic persistence as a driver for the evolution of resistance.*** For a long time, it remained unclear whether antibiotic persistence and resistance are alternative strategies for survival during antibiotic therapy, or whether these two processes are linked. Recent studies have shown that they are indeed linked. It is important to re-emphasize that antibiotic-resistant clones can emerge by two main means: mutation or acquisition of genes by HGT. Antibiotic persistence can promote both processes.

The role of antibiotic persistence in the emergence of resistance mutations was first demonstrated in vitro. Intermittent exposure to stressors such as antibiotics promotes the emergence of mutations that enhance antibiotic persistence[20,21,24,26]. Moreover, the levels of persistence and the rate of emergence of resistance are positively correlated in clinical isolates of *P. aeruginosa*[107] and *E. coli*[75]. Importantly, an in vitro study of *E. coli* demonstrated a direct causal link between persistence and the emergence of resistance[20]. Mutations increasing tolerance allowed a larger fraction of bacteria to survive antibiotic therapy. This link was also recently observed to occur in patients[78]. A higher number of survivors increased the chance that resistance mutations would emerge. In addition, the stress itself, which also induces both persistence and mutator phenotypes[18,75,108], may also increase the mutation rates. Accordingly, another study of *E. coli* proposed a second mechanism by which persistence can promote the emergence of resistance mutations: higher mutation rates in persisters themselves[75]. This higher rate should further boost the rise of antibiotic-resistant mutants[75]. Thus, the convergent roles of stress signalling, such as the stringent response through (p)ppGpp and the SOS response, in both persister formation and error-prone DNA repair[18,75] will promote the emergence of resistant bacterial strains.

Recently, we found that persisters can also promote the spread of antibiotic resistance genes by HGT. In mice, *S.* Typhimurium forms substantial numbers of persister cells that survive ciprofloxacin or ceftriaxone treatment in the gut tissue or at systemic sites[42–45]. These persisters formed reservoirs for the resistance plasmids that they

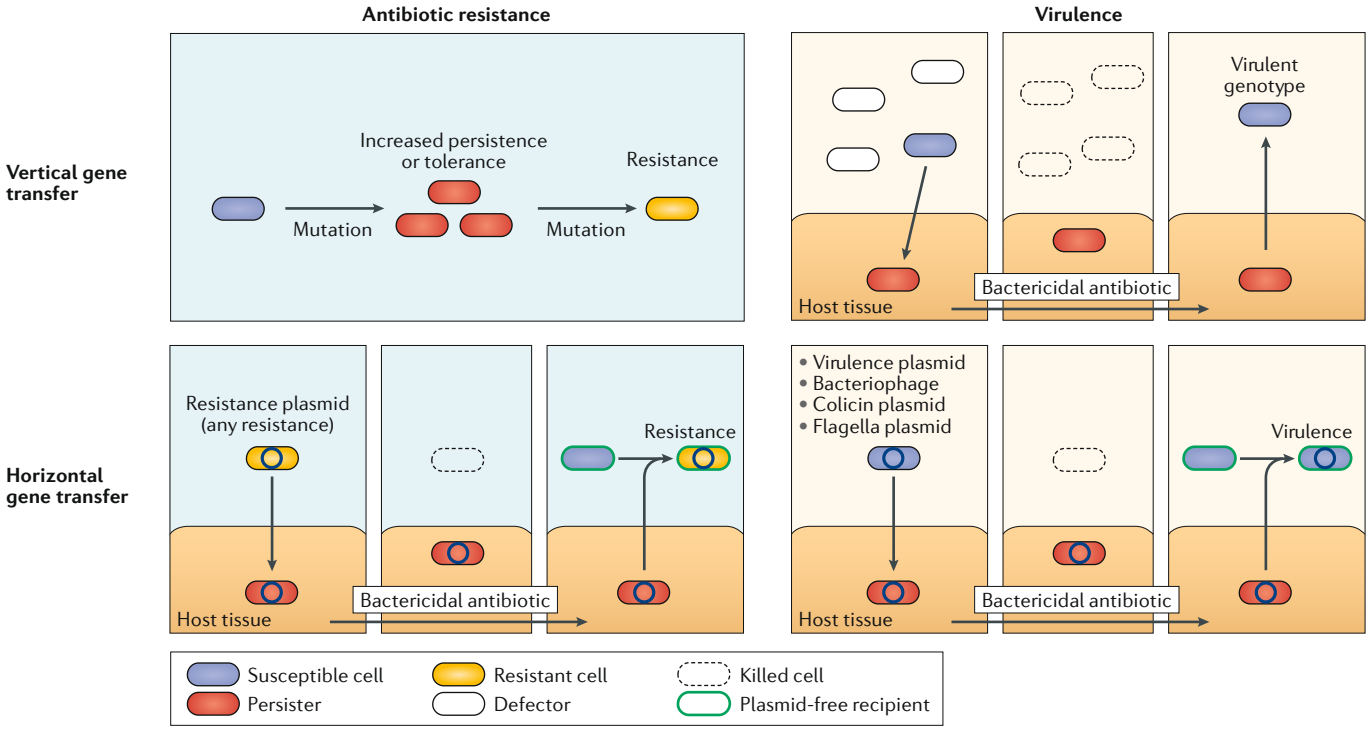


Fig. 3 | **Evolutionary consequences of persistence.** Persisters (red) can fuel the evolution of both antibiotic resistance and virulence of bacteria. At the chromosomal level (vertical gene transfer), persisters increase the chance of a mutation leading to resistance (orange) (top left panel)[20]. In the case of *Salmonella enterica* subsp. *enterica* Typhimurium, invasion-capable *S.* Typhimurium (filled rods) can survive antibiotic therapy in host tissues as persisters, whereas defectors (white rods; non-invasive) cannot (top right panel)[42]. Through antibiotic treatment, persisters enable the selection for virulent clones. At the level of mobile genetic elements (horizontal gene transfer), persisters form long-term reservoirs that can store antibiotic resistance plasmids, which can be donated to plasmid-free recipients (illustrated with green outline) after antibiotic treatment (bottom left panel)[44]. We propose that a similar phenomenon can occur for mobile genetic elements containing virulence or other fitness determinants, such as colicin plasmids or bacteriophages (bottom right panel). Antibiotic susceptible bacteria are shown in blue, persisters in red and resistant bacteria in orange; mobile genetic elements are depicted as black circles. Killed bacteria are shown as dotted, hollow rods.

carried. When such persisters reseeded the gut lumen, they could efficiently transfer the resistance plasmid to new Enterobacteriaceae[44]. Thus, by increasing the chance of co-occurrence of plasmid donors and recipients in the gut, the persister reservoirs may help to explain why Enterobacteriaceae harbour such vast numbers of resistance and virulence plasmids or obtain mobile genetic elements over time during persistent infections[109–111]. Besides selecting for persister survival, the antibiotic therapy in our mouse model had additional interesting effects. After the cessation of antibiotic treatment, the relapsing *S.* Typhimurium cells could bloom in the gut lumen (owing to the antibiotic-mediated microbiota disruption[42,44,112]). These high densities of donors and recipients could further promote the plasmid spread[113,114]. Indeed, plasmid transfer was so efficient that no antibiotic selection was needed to replace 99% of the recipients by transconjugants within 1–3 days[44].

Importantly, the inverse scenario is also true: *S.* Typhimurium persisters residing in tissues could also serve as recipients for plasmids from gut-resident commensal *E. coli*[44]. This scenario begs the question of whether persistent bacteria could, in general, act as a 'storage device' for mobile genetic elements, transiently sampling the environment and acquiring new material as a snapshot of a current process occurring in the gut lumen. Such questions provide interesting ecological and evolutionary implications to persister biology and the spread of antibiotic resistance and should be addressed.

Finally, it should be noted that persistent infection per se (that is, survival within the host when no antibiotics are applied) should similarly boost the spread of antibiotic resistance (by HGT), as discussed above for antibiotic persistence. *S.* Typhimurium can persist in the tissues of infected hosts for very long periods of time (for example, over 200 days in mice)[36], and rare events of gut-luminal reseedings followed by plasmid transfer suffice for high levels of plasmid spread[44]. Therefore, it is possible that the indirect evolution of *S.* Typhimurium persistence through its intracellular lifestyle has led it to become a potent long-term spreader of mobile genetic elements. Possibly, this pertains not only to antibiotic resistance determinants but also to virulence factors encoded by mobile genetic elements (FIG. 3). As HGT requires two different bacterial participants, through establishing a reservoir, persisters prolong the timescale of interactions between different bacteria (that is, they increase the co-occurrence of bacteria)[44]. It remains to be seen whether similar interactions can occur in other environments in which persisters can be observed

interacting with other bacteria, such as in biofilms, in sewage tanks or during clinical persistence of other pathogens.

***Persistence and virulence.*** Virulence and persistence seem to be intimately linked, particularly when one considers the intracellular lifestyle of pathogens such as *S.* Typhimurium, which requires specific virulence determinants[33]. For some pathogens in which persistence in the clinic has been described, evolution towards commensalism has been observed, potentially because the relatively silent lifestyle of persisters diverts energy to survival rather than expression of virulence factors[33]. This trajectory has been suggested for lung infections by *P. aeruginosa*[53,54,115] and urinary tract infection by *E. coli*[116].

*S.* Typhimurium provides another example of evolution towards virulence attenuation. Diarrhoea and the maintenance of the virulent genotype rely on the cooperation between two different *S.* Typhimurium phenotypes that form in the gut lumen. One subpopulation engages in the costly expression of flagella and the virulence determinants SPI-1 and the SPI-4 adhesin SiiE to swim towards the epithelium, infect the mucosa and thereby elicit inflammation[96,112,117–122]. The host inflammatory defence reduces pathogen loads in the gut tissue[118,123], but also changes the milieu in the gut lumen and thereby helps *S.* Typhimurium to compete with the microbiota[93,124–128]. As inflammation is a public good and SPI-1 expression is associated with a cost, attenuated mutants that profit from the inflammation without expressing SPI-1 rapidly emerge; these mutants are termed 'defectors'[129]. Because defectors grow more rapidly than the cost-burdened SPI-1-expressing cells, they can rapidly overtake the gut-luminal pathogen population, leading to eventual population collapse owing to the regrowth of the microbiota after inflammation has been cleared[129,130]. The ability to colonize host tissues (by SPI-1 and SPI-2), which leads to indirect persister formation (discussed above), ensures that the persisters are in fact reservoirs of virulent, wild-type *S.* Typhimurium cells that survive for extended periods within host tissues and can be transmitted over long periods of time to new hosts[33,35,36,42–44]. Indeed, this has been experimentally demonstrated by treating defector-overridden mice with ciprofloxacin to enrich for tissue-lodged persisters[42]. After the cessation of treatment, virulent *S.* Typhimurium reseeded the gut lumen and triggered disease in mice after transmission[42]. Therefore, *S.* Typhimurium persisters can promote the maintenance of virulence and transmission upon antibiotic treatment.

Given the recent implication of persisters in the spread of resistance by HGT, it is likely that persisters could also be reservoirs of mobile genetic elements that contain virulence factors. For example, efficient transfer of a virulence-encoding temperate phage between different *S.* Typhimurium strains was demonstrated in vivo[131,132]. In many bacterial pathogens, virulence factors are encoded on phages or conjugative plasmids[133,134]. Therefore, to what extent persisters can drive the spread of virulence factors and evolve new clones with increased virulence in the process should be investigated. Additionally, there are many plasmids that contain interbacterial competition factors, such as colicin plasmids[114,135]. The P2 plasmid encodes such a colicin and was shown to spread rapidly between bacteria by a process facilitated by persisters[44,114]. In this case, the plasmid recipients in the gut lumen were not sensitive to the plasmid-encoded colicin. However, it is possible that persisters can promote the spread of colicin plasmids (and the associated colicin resistance genes) to bacteria in the gut, which in turn would become more fit than their plasmid-free counterparts[114]. This process may have implications for the ecological dynamics of microbial species in the gut, particularly Enterobacteriaceae. Such investigations could be important to unravel the mechanisms that lead to ecological succession of *E. coli* strains in humans and animals[136–138].

## Influencing factors

With each experimental observation, there are clear limitations in the conclusions that can be drawn. These limitations are based on experimental constraints. However, in spite of these constraints, we can uncover key factors affecting the experimental outcome, which could eventually help inform clinical decisions. For example, there are several modulating factors that influence how persistence is selected for, and how persistence affects bacterial fitness, including the strength and duration of the selective force, and the population size and structure that are subject to selection. These factors may be of practical importance when designing anti-persister therapies.

***Strength and duration of selection.*** An obvious factor that influences the evolution of tolerance or persistence is the strength (that is, the concentration) and duration of the selective force. It is conventional clinical wisdom that in the case of treatment failure with antibiotics in patients, lack of adherence to therapy can be an important factor promoting the emergence of resistance[61,139]. Indeed, evolutionary experiments are highly context dependent; a strict regimen of intermittent selection by antibiotics led to the emergence of both persistence[21,24,26] and, ultimately, resistance[20] after a certain number of cycles. These studies approximate clinical treatment regimens of once-daily antibiotic therapy. However, there is a growing collection of theoretical and clinical studies suggesting that the dose, timing and combination of antibiotics may strongly influence the evolution of resistance[140]. Based on in vitro experimental evolution, it is probable that similar concepts apply for the evolution of persistence in vivo.

However, in some cases, the strength and duration of an antibiotic treatment may have less of a role. For example, let us consider the formation of reservoirs of persisters leading to resistance plasmid spread[44] or transmission of a virulent genotype[42]. In *S.* Typhimurium-infected hosts, such tissue reservoirs of invasive bacteria can last for weeks or months[36,43,44]. Therefore, one could speculate that whether the duration of treatment varies by days or weeks would have a minor role. Indeed, persisters may continue to facilitate transmission or plasmid spread for long periods after the cessation of treatment. This consideration may also be relevant for other pathogens, such as *S. aureus*, *E. coli*

Defectors
Mutants that do not pay a cost associated with production of a public good, as they do not produce it, but can still profit from the public good produced by others. This destabilizes cooperation in bacteria, as defectors are more fit (given the presence of the public good) and will therefore outcompete cooperators. Defectors can also be called 'cheaters'.

Persistence as a social trait
An ecological explanation for persistence in which subpopulations of metabolically inactive, slow-growing and fast-growing cells exist so that nutrient competition is decreased among cells. This cooperative behaviour increases the growth efficiency at a population scale.

Persistence as stuff happens
An explanation for the existence of persistence in which persistence occurs owing to errors in cellular processes. Such errors occur in only a minor fraction of cells at a given time and could explain why metabolically inactive, survival-ready cells emerge in populations of otherwise susceptible growing cells.

and *P. aeruginosa*, in which long-lasting, persister-driven recalcitrance leads to long-term antibiotic failure. Additionally, it is likely that besides the antibiotic persisters, the antibiotic-susceptible bacterial cells (which may, in fact, be more numerous than the antibiotic persisters) also contribute as long-term pathogen reservoirs. In the absence of antibiotics, the mix of susceptible and persistent bacterial cells promotes the maintenance of the pathogen and the chance for de novo emergence of new mutations or for HGT within a given host for extended periods of time.

***Population size and structure.*** The impact of the strength and duration of selection is also affected by the population size of bacteria that survive during antibiotic treatment. Facilitation of resistance by increased tolerance (or increased fractions of persisters) has been linked to an increase in the population size of bacteria that survive antibiotic treatment[20]. This effect is likely dependent on the population size that existed prior to antibiotic treatment.

A second example relates to the implication of persistence in forming long-term reservoirs for HGT or transmission[42,44]. Here, the population size has a twofold effect.

First, in *S.* Typhimurium, the spread of plasmids from tissue-lodged persisters was heavily dependent on the reservoir size of plasmid-bearing persisters in the host tissues. Plasmid spread was diminished when invasion-deficient pathogen mutants were used or when hosts were vaccinated before infection[44]. This effect would likely also apply to transmission of virulent phenotypes by persisters reseeding the gut[42]. Moreover, the presence of intrinsic *E. coli* was recently shown to reduce diet-shift elicited blooms of *S.* Typhimurium[141]; this reduction of blooms would also likely reduce persister reservoir sizes, as the population able to actively invade into host tissues and form the persister state is substantially reduced.

Second, HGT itself is heavily dependent on the population density of both interacting partners (donors and recipients)[142]. The ecological niche in the gut provides no exception; using mouse models, plasmid transfer between Enterobacteriaceae is prodigal during blooms elicited by inflammation, diet shifts or microbiota communities with limited colonization resistance, but is meagre in the face of colonization resistance that limits donor and recipient densities[114,141,143]. Therefore, in this case, the density of the HGT interacting partners of persisters also has a major role in facilitating the spread of mobile genetic elements. Although Enterobacteriaceae in the microbiota of humans and animals can reach high densities[136], it will be important to address persister-mediated spread of plasmids in more natural situations, such as during clinical studies or by assessing livestock.

Population structure also likely has a role. Heavily structured populations, for example, in biofilms, have been strongly linked with increased plasmid transfer[144]. Considering that biofilms are riddled with persisters and promote plasmid stability[18,144], it is possible that these structured populations are particularly potent reservoirs for persistent bacteria. Furthermore, one might consider an infected host as a structured population within a community of several infected hosts. Given the hypothesis of 'persistence as a social trait', this idea becomes a particularly important ecological consideration[145] (BOX 1). In this case, persistence is suggested to be a cooperative trait selected for by rules of kin selection[146,147], in which genetically related individuals cooperate to limit resource competition (that is, persisters do not use scarce nutrients, which enables the rest of the population to grow)[145]. Highly structured homogeneous populations achieved by rapid clonal growth or growth in isolated hosts support this behaviour. Heterogeneous populations, for example, in which defector bacteria with lower frequencies of persister formation emerge (benefiting from reduced resource competition from high-persister forming bacteria), destabilize this cooperative behaviour[145].

Therefore, manipulating pathogen population size or structure by foods, virulence inhibitors or vaccination may offer practical opportunities for limiting the emergence of pathogens with increased virulence or additional antibiotic resistance determinants.

## Conclusions and perspectives

An increasing amount of research aims to elucidate the mechanisms of persister formation. The bulk of these studies has been conducted in vitro[6,11,18,20,21,24,75], which provides important insight into potential anti-persister therapies. In general, anti-persister therapies are grouped at targeting persister formation, direct killing of persisters or sensitizing persisters to antibiotics; reasonable advances have been made for all three strategies[148]. For example, persister formation in the stationary phase can be reduced by the inhibition of respiration systems used by *E. coli* in the stationary phase[149], efflux pump

Box 1 | **Explanations for spontaneous persistence**

Alternative explanations for antibiotic persistence have been proposed. These hypotheses might explain the baseline of spontaneous persistence. Levin et al. coined the 'persistence as stuff happens' hypothesis[157] to explain persistence as transient and random errors in basic cellular processes, leading to a consistent mixture of optimally growing and functioning bacteria and a subpopulation of slower-growing, survival-ready cells. Levin et al. state that even without selection for persistence itself, during stressful conditions that are toxic to the fast-growing subpopulation, the transiently 'glitched' subpopulation would be positively selected[157]. This explanation would fit particularly well for spontaneous persistence. Under stressful conditions when error rates of cells are increased, persistence as stuff happens would also contribute to triggered persistence. An alternative ecological explanation for the evolution of persistence is 'persistence as a social trait'[145]. In this case, a subpopulation engaging in persistence benefits the overall population by a decrease in competition for nutrients, which would be particularly important in the stationary phase[145]. The cooperation between the persistent subpopulation and the growing subpopulation decreases the amount of nutrients used by the total population. Persistence as stuff happens and persistence as a social trait would also satisfy the typical biphasic killing curves of persistence.

inhibitors have shown promise in reducing antibiotic tolerance in *Mycobacteria* spp.[150], persister populations of *E. coli* or *S. aureus* can be sensitized to aminoglycoside treatment by specific metabolites[151] and direct killing of persisters has been observed in *S. aureus* biofilms by overactivating a cellular protease[152] or by the addition of bacteriophages[153]. However, there are many aspects of both persister biology and the evolutionary implications of persistence that are not captured using in vitro systems. Further, although studies with clinical samples are excellent for hypothesis generation, they often cannot be used to study persistence mechanisms in vivo. Along with others, we advocate for studying both resistance evolution[154] and persister biology[6] using adequate host model systems, such as during infection.

Combining clinical studies, infection biology and research with evolution of bacterial pathogens in relevant mouse model systems has uncovered links between persistence and both virulence and antibiotic resistance[42,44]. Importantly, such work has determined a role for vaccination in preventing the establishment of persister reservoirs[44]. Mucosal vaccination against *S.* Typhimurium generates high-avidity IgA in the gut that enchains growing bacteria, limiting plasmid transfer, disease and the establishment of persister reservoirs in host tissues[44,155,156]. Such vaccinations should be safely applicable to livestock to limit resistance plasmid spread and transmission of virulent pathogens.

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