Persistent bacterial infections and persister cells

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Abstract | Many bacteria can infect and persist inside their hosts for long periods of time. This can be due to immunosuppression of the host, immune evasion by the pathogen and/or ineffective killing by antibiotics. Bacteria can survive antibiotic treatment if they are resistant or tolerant to a drug. Persisters are a subpopulation of transiently antibiotic-tolerant bacterial cells that are often slow-growing or growth-arrested, and are able to resume growth after a lethal stress. The formation of persister cells establishes phenotypic heterogeneity within a bacterial population and has been hypothesized to be important for increasing the chances of successfully adapting to environmental change. The presence of persister cells can result in the recalcitrance and relapse of persistent bacterial infections, and it has been linked to an increase in the risk of the emergence of antibiotic resistance during treatment. If the mechanisms of the formation and regrowth of these antibiotic-tolerant cells were better understood, it could lead to the development of new approaches for the eradication of persistent bacterial infections. In this Review, we discuss recent developments in our understanding of bacterial persisters and their potential implications for the treatment of persistent infections.

Numerous bacterial infections are able to persist in the host for long periods of time, even during antibiotic treatment. Several factors in both the pathogen and host are thought to contribute to the establishment and maintenance of persistent infections, including antibiotic resistance and immunosuppression. Another phenomenon that is steadily gaining recognition for its role in persistent infections is the presence of persister cells (BOX 1). Persister cells are thought to be ubiquitous among bacterial species and have been well described in *Escherichia coli*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Staphylococcus aureus*[1–3]. Persisters cause antibiotic treatment failure, are selected for following repeated doses of antibiotics[4,5] and have been shown to lead to the emergence of antibiotic resistance[6,7]. Therefore, persisters are a major public health concern. Persisters were first described soon after the discovery of antibiotics[8,9]. The term was first used by Joseph Bigger in 1944 after he discovered a culture of *S. aureus* that could not be sterilized with high doses of penicillin. He hypothesized that a minority of cells survived antibiotic treatment owing to their lack of growth rather than a heritable resistance mechanism[9]. However, this hypothesis was not confirmed for 60 years, until a study that used microfluidics and live imaging showed that the bacterial cells that survived antibiotic treatment were not growing before the addition of antibiotics[10]. Many studies have since corroborated this observation (as reviewed in REF. 1). In addition to growth arrest or slow growth[1,11,12], a decrease in metabolic activity is often associated with the persister phenotype[13]. However, persisters are not necessarily metabolically inactive[14].

In recent years, there have been numerous studies that have heightened, but also challenged, our understanding of how multidrug-tolerant persisters form and have given us insights into the mechanisms that are responsible for the regrowth of persisters. Advances have been made in the technologies that are used to study persister cells and several approaches for killing them have been proposed (BOX 2). The inherent ability of persister cells to survive antibiotic treatment, without being genetically resistant, is likely to be a key factor in many persistent bacterial infections. However, despite the relevance of persisters to infection, the bulk of research into persister biology is still carried out in laboratory strains that are cultured in unphysiological nutrient-rich medium. In this Review, we discuss recent advances in our understanding of the formation and regrowth of persisters in the context of infection.

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Box 1 | **Definition of field-specific terms**

Persistence
The ability of bacteria to remain viable in the host for a prolonged period of time. Not to be confused with bacterial persisters.

Bacterial persisters
Cells that represent a subset of antibiotic tolerance; persisters are a subpopulation of slow-growing or growth-arrested bacterial cells that have a decreased susceptibility to killing by bactericidal antibiotics within an otherwise susceptible clonal population, owing to a low target activity or low antibiotic uptake that is induced by stress.

Antibiotic resistance
The ability of a population of bacteria to grow in the presence of antibiotics, owing to an acquired genetic modification that enables the degradation or export of the antibiotic, or modification of the antibiotic target.

Phenotypic resistance
A form of antibiotic resistance; the ability of a subpopulation of bacterial cells to grow in the presence of antibiotics within a clonal and otherwise susceptible population, owing to phenotypic variation that enables the degradation or export of the antibiotic, or modification of the antibiotic target.

Antibiotic tolerance
The reduced susceptibility of a population of bacteria to killing by bactericidal antibiotics, owing to low target activity or low drug uptake, often associated with slow growth or reduced metabolism induced by stress.

Bacterial persistence in the host

Persistent infections

Asymptomatic (subclinical) or symptomatic persistent bacterial infections can take place in various tissues within the host (TABLE 1) and are associated with antibiotic treatment failure[15,16]. Asymptomatic persistent infections are regularly undiagnosed and can be caused by growing or growth-arrested bacteria. Subclinical persistent infections caused by actively growing bacteria are not only a burden on the host but also have the potential to spread to others and cause disease. For example, typhoid fever, which is caused by infection with *S. enterica* subsp. *enterica* serovar Typhi, can lead to a state of asymptomatic carriage in 2–5% of patients, with large amounts of bacteria being shed from the gallbladder[17]. This condition can take four times longer to treat than acute typhoid fever and substantially increases the risk of gallbladder cancer[18]; treatment may fail even after this long course of antibiotics, requiring the removal of the gallbladder and additional antibiotic therapy[19]. Infection with *Helicobacter pylori*, which is associated with an increased chance of developing stomach ulcers[20] and gastric cancers[21,22], also leads to the shedding of bacteria. Asymptomatic persistent infections can also be caused by latent growth-arrested bacteria and are associated with relapses of acute symptomatic infections. For example, 10% of individuals who are infected with latent *M. tuberculosis* following initial acute infection will relapse during their lifetime[23]. Typhoid fever recurs in 15% of patients following antibiotic treatment[24], and following a urinary tract infection (UTI) caused by uropathogenic *E. coli* (UPEC), 27% of women will suffer a relapse[25]. Several other bacterial infections, including streptococcal tonsillitis[26], tend to recur after antibiotic treatment and are likely to have a period of latency in the host.

Symptomatic persistent infections are characterized by a long period of clinical manifestations, often despite treatment with antibiotics[16]. They can be life threatening and often follow the use of indwelling medical devices or the suppression of immunity during chemotherapy or infection with HIV. Persistent infections that are associated with indwelling medical devices account for around half of all nosocomial infections and are notoriously recalcitrant to drugs[27]. Acute infection with *M. tuberculosis* can take several months to treat with antibiotics[23], and lung infections in patients with cystic fibrosis, which are often caused by *P. aeruginosa, Burkholderia cepacia, S. aureus* and *H. pylori*, become increasingly difficult to treat over time, despite remaining apparently susceptible to antibiotics in laboratory tests[28]. The long courses or repetitive use of antibiotics owing to recalcitrant or relapsing infections will not only have negative effects on patient health, through side effects and depletion of the resident microbiota[29], but also increase the selection of antibiotic-resistant strains. Therefore, there is a need for more effective strategies to treat persistent infections.

Why do infections persist?

Ineffective clearance by the host. In the absence of antibiotic treatment, a persistent infection is the result of the failure of the host immune system to clear the causative organism. This may be due to an inability of the immune system to detect the pathogen. Some bacteria have evolved strategies to passively avoid detection by the host; for example, *Borrelia* spp. vary the expression of surface antigens during infection[30]. Pathogens can also modulate host immune responses, thereby triggering an inappropriate anti-inflammatory response, thus decreasing the chance of pathogen clearance. For example, *M. tuberculosis* and *Listeria monocytogenes* induce the production of interleukin-10 (IL-10) in macrophages to suppress the interferon-γ response (IFNγ response) that otherwise restricts bacterial growth[31]. Moreover, many pathogens, including *M. tuberculosis*, *Legionella pneumophila* and *Salmonella* spp., avoid degradation following phagocytosis by host immune cells by inhibiting the maturation of phagosomes[32]. Pathogens can also interfere with the adaptive immune response; for example, the *S.* Typhimurium *Salmonella* pathogenicity island 2 (SPI-2) type III secretion system (T3SS) effector SteD was recently shown to inhibit MHC class II antigen presentation in dendritic cells[33], probably influencing the host T cell response in favour of the pathogen. These are only a few examples of the diverse strategies that bacteria use to evade host immunity; this expansive topic has been well reviewed elsewhere (see REFS 34,35).

Even if the host detects the pathogen and an antibacterial response is mounted by the immune system, eradication of the pathogen is not guaranteed. For example, *H. pylori* escapes clearance despite inducing high levels of inflammation in the stomach epithelium[36]. This is dependent on many factors, including adherence, cell motility, the detoxification of reactive oxygen species (ROS) and toxin secretion[37]. The formation of biofilms, which involves the secretion of polysaccharides and DNA by bacteria to form a protective extracellular

Nosocomial infections
Infections acquired when under medical care, also known as hospital-acquired infections (HAIs).

Interleukin-10
(IL-10). A cytokine that suppresses the interferon (IFN)-mediated transcriptional response.

Interferon-γ response
(IFNγ response). The transcriptional changes that take place in a cell due to the detection of the cytokine interferon-γ (IFNγ), some of these changes increase the antibacterial capabilities of phagocytes.

Type III secretion system
(T3SS). A protein needle-like appendage that is used by bacteria to translocate effector proteins into eukaryotic cells to manipulate host cellular processes.

MHC class II antigen presentation
The process of presenting antigen bound to MHC class II on the cell plasma membrane for recognition by cells of the adaptive immune system.

Granulomas
Organized groupings of macrophages and other cell types around foreign material that the immune system is unable to eliminate.

Bactericidal antibiotic
An antibiotic that kills bacteria, as opposed to a bacteriostatic antibiotic, which only inhibits growth.

Salmonella-containing vacuole
(SCV). A membrane-bound compartment that resembles that of a modified late endosome, in which *Salmonella* spp. typically reside within host cells.

Fluoroquinolones
A class of antibiotics that target DNA gyrase activity to induce the formation of lethal double-stranded breaks in bacterial DNA.

matrix, has also been linked to the persistence of many infections[38]. Biofilms can block complement-mediated and cell-mediated killing in many persistent infections, such as those that are associated with indwelling devices[27,39,40]. Furthermore, *M. tuberculosis* cells can be effectively 'walled off' by macrophages in lung tissue to form granulomas. This restricts the growth of the bacteria, but the pathogen is not cleared. If host immunity is subsequently suppressed, bacterial growth may resume and result in a relapse of acute tuberculosis[41].

Ineffective clearance by antibiotics. Antibiotic treatment failure can be due to several factors, such as low patient compliance and poor pharmacokinetics of drugs in infected tissues[42,43]. In addition, antibiotic resistance that is acquired through genetic adaptation or horizontal gene transfer may enable the survival and growth of bacteria in the presence of antibiotics, even at concentrations that are toxic for the host[44]. In the absence of resistance, bacteria may survive drug treatment through antibiotic tolerance[45] (FIG. 1a). Tolerance is a decrease in the susceptibility of a bacterial population to killing by a bactericidal antibiotic. Tolerance can also be described as an increase in the time it takes for an entire bacterial population to be killed at a given concentration of antibiotic[45]. Slow growth may increase the duration of antibiotic exposure that is required to kill the population, this is probably owing to a reduction in the activity of metabolic drug targets and decreased antibiotic uptake. Antibiotic resistance and tolerance can either occur on the level of a whole population of bacteria or in a subpopulation through phenotypic heterogeneity, known as phenotypic resistance or persister formation, respectively. Persisters can be multidrug tolerant, but may differ in their sensitivity to different antibiotics. They are slow-growing or growth-arrested bacteria that are able to re-establish growth once cellular stress pathways are deactivated, but they regain sensitivity to antibiotics once growth resumes[1–3,46]. Homogenous populations of bacteria that are resistant, tolerant or susceptible to antibiotics theoretically exhibit differential killing kinetics during treatment *in vitro* (FIG. 1a); however, a clonal population of bacteria is rarely entirely homogenous and will respond to antibiotic treatment in a heterogeneous manner. For example, *S.* Typhimurium cells that are cultured in nutrient-rich media have classic biphasic killing kinetics following the addition of the antibiotic cefotaxime, because of the simultaneous presence of antibiotic-tolerant persister cells in addition to the bacterial cells that are sensitive to the drug (FIG. 1b). Antibiotic-susceptible bacteria die rapidly after the addition of cefotaxime, which results in a large initial decrease in colony forming units (CFU), whereas persister cells remain viable, which is represented by a plateau in the killing kinetics. *S.* Typhimurium cells that are released from macrophages 30 min after phagocytosis and are then cultured in nutrient-rich media in the presence of cefotaxime have similar biphasic killing kinetics. However, the persister fraction is much larger, owing to the activation of cellular stress responses that are induced by the acidic and nutrient-limiting conditions within the *Salmonella*-containing vacuole (SCV)[14]. A clonal population of *S.* Typhimurium that is treated with antibiotics while inside macrophages is even more heterogeneous; the total population replicates at a slower rate compared with growth in nutrient-rich medium and is therefore more tolerant to antibiotic treatment. In addition to these slow-growing tolerant cells, there is a subpopulation of growth-arrested persister cells that are even less susceptible to the drug. In this case, a more gradual decrease in the number of viable cells is observed until only the persister fraction remains[14]. During infection of host tissues, antibiotic-tolerant slow-growing bacteria and growth-arrested persisters are likely to exist simultaneously. Variable sensitivity to antibiotics as a consequence of differential growth kinetics has been well described during antibiotic treatment of murine *S.* Typhimurium infection[47]. Growth-arrested *S.* Typhimurium cells were found to be the least susceptible to killing by fluoroquinolones. Interestingly, the largest surviving proportion of cells had an intermediate rate of growth, which enabled the bacteria to grow without being killed rapidly by the antibiotic. One could speculate that a slow-growing tolerant population of bacteria is of concern in a chronic infection that is associated with treatment failure, whereas growth-arrested persisters can provide a source of bacterial regrowth that results in the recurrence of clinical symptoms after an

Box 2 | **Methods for studying persisters**

The first methods used to study persisters relied on antibiotics that selected for the insensitive cells[52]. However, in recent years, fluorescent reporters have enabled a much larger repertoire of techniques[133] to be developed and applied to the study of persisters, without the use of antibiotics.

Fluorescent reporters have been used to measure the metabolic activity or growth of persisters. A fluorescence dilution reporter plasmid, which provides an inverse measure of bacterial proliferation on the basis of fluorescence, was used to distinguish growing and growth-arrested *Salmonella enterica* subsp. *enterica* serovar Typhimurium cells within macrophages[14,134]. A GFP reporter and fluorescence-activated cell sorting (FACS) have also been used to isolate persister cells for transcriptomic analysis[79]. Furthermore, metabolically active *Mycobacterium tuberculosis* persisters were monitored *in vitro* and during murine infections using an unstable GFP rRNA gene fusion[135].

Microfluidics has been used in combination with fluorescent reporters (for example, fusions with toxin–antitoxin promoters[60] or metabolic reporters[135]) to study persisters *in vitro*.

Transcriptomics has been carried out using microarrays on enriched persister fractions that were isolated using fluorescent reporters[81]. However, as next-generation DNA sequencing is becoming more affordable, RNA sequencing (RNA-seq) is becoming the method of choice for transcriptomics. RNA-seq has substantially increased the sensitivity and the dynamic range compared with microarrays[136], making it particularly useful for the study of persisters, which are often a small subpopulation of cells that probably synthesize low levels of RNA.

Potential future developments for persister research include the design of improved techniques that are able to identify the factors that are associated with, and specific to, the formation of persisters or persister regrowth. Current methods that use colony forming units (CFU) to measure levels of bacterial persisters often cannot differentiate between levels of persister formation or levels of persister regrowth. One major problem with monitoring persister regrowth by FACS or microfluidics is that a sample can quickly accumulate numerous early-regrowing bacteria, which prevents any observations of late-regrowing cells. The ScanLag technique has the potential to be invaluable for monitoring the regrowth of persisters. The ScanLag method uses time-lapse imaging to monitor the appearance and growth of bacterial colonies on agar plates over time[137] and has been used to study the emergence of antibiotic-tolerant and antibiotic-resistant cells in evolution experiments[7,51].

Table 1 | **Examples of bacterial species that cause persistent infections**

Bacterial species	Site of infection	Form of persistent infection
Mycobacterium tuberculosis[36]	Respiratory	Symptomatic or asymptomatic (latent)
Pseudomonas aeruginosa[53]	Respiratory	Symptomatic
Burkholderia cepacia[147]	Respiratory	Symptomatic
Haemophilus influenzae[148]	Respiratory	Symptomatic
Helicobacter pylori[36]	Gastrointestinal	Asymptomatic (active)
Brucella abortus[149]	Gastrointestinal	Asymptomatic (active or latent)
Escherichia coli[150]	Gastrointestinal	Symptomatic
Clostridium difficile[151]	Gastrointestinal	Symptomatic
Salmonella enterica subsp. *enterica* serovar Typhi or S. Paratyphi[36]	Systemic	Asymptomatic (active or latent)
Nontyphoidal invasive *Salmonella* spp.[152]	Systemic	Asymptomatic (active or latent)
Borrelia burgdorferi[30]	Systemic	Symptomatic
Staphylococcus aureus[153]	Systemic	Symptomatic
Streptococcus pyogenes[50]	Systemic	Asymptomatic (latent)
Chlamydia trachomatis[154]	Sexually transmitted infection	Asymptomatic (active)
Treponema pallidum[155]	Sexually transmitted infection	Asymptomatic (latent)
Uropathogenic *E. coli*[63]	Urinary tract infection	Asymptomatic (latent)

apparently successful course of treatment with antibiotics. It is important to keep in mind that the reasons for antibiotic treatment failure in the host are complex and may be the result of a combination of the interactions between the pathogen and host immunity, and antibiotic resistance and tolerance on both the population and subpopulation levels.

Persister cells and persistent infections

In recent years, evidence that bacterial persisters have a role in the relapse and recalcitrance of infections has been accumulating. Monitoring SNPs between isolates of *S.* Typhimurium from cases of invasive non-typhoidal salmonellosis (iNTS) showed that in cases of recurrent fever, 78% were caused by the same genovar as the original infection[48]. Similar observations were made by sequencing UPEC strains from patients with UTIs[49] and *Streptococcus pyogenes* strains from patients with streptococcal tonsillitis[50]. These findings indicate that the bacteria that caused these infections were never cleared from the host during treatment with antibiotics.

Antibiotic tolerance is generally considered to be the result of non-heritable phenotypic changes in bacteria; however, genetic mutations may also indirectly increase tolerance by slowing growth, metabolism or the uptake of drugs. For example, mutations that led to an increase in the lag time of bacteria were found to increase antibiotic tolerance[51]. Similarly, mutations can increase the probability of persister formation in a clonal population. These are known as high-persistence (hip) mutations and were first described in *E. coli* in reference to the *hipA7* mutation[52]. Clinical isolates of *P. aeruginosa* from lung infections in patients with cystic fibrosis are more likely to be hip mutants if obtained later in infection compared with isolates obtained earlier in infection[53]. *E. coli* hip mutants have also been isolated from patients with UTIs[54]. Similarly, periodic treatment of *Candida albicans* with amphotericin B was found to select for hip mutants[55], which indicates that the persister phenomenon may also be an important factor in the recalcitrance of fungal infections. Furthermore, in *E. coli* and *S. aureus* cells, hip mutants were found after periodic treatment with antibiotics *in vitro*[4,5,52]. These findings suggest that the persister phenotype may be an important first line of defence against antibiotics before resistance is acquired.

The uptake of bacteria by host cells has been linked to an increase in the relative number of persister cells in a population. For example, *S.* Typhimurium and *M. tuberculosis* both increase the formation of persisters inside macrophages[14,56]. In addition, several studies have described a link between the presence of persisters and biofilm formation[55,57–61]. Concurrently, evidence is building that suggests that the ability of bacteria to reside intracellularly and the formation of biofilms are associated with persistent infections[14,16,38,62,63]. *P. aeruginosa* forms biofilms during lung infections in patients with cystic fibrosis[64], whereas *H. pylori* forms biofilms and adopts an intracellular lifestyle during infection[65,66]. UPEC can form biofilms on the bladder epithelium, as well as quiescent intracellular reservoirs that could contribute to relapsing infections[63]. *Salmonella* spp. can form biofilms on gallstones[67] and *S.* Typhimurium persisters have been found residing within macrophages in the spleens of infected mice six days after the initial infection[14]. Considering the *in vitro* evidence that there are high levels of persister formation in biofilms and inside host cells, combined with the prevalence of biofilm formation and the intracellular lifestyles of bacteria during persistent infections, the formation of persisters is probably a substantial factor in the relapse and recalcitrance of these infections.

Genovar
A classification used for differentiating between strains of the same serovar that differ substantially in their genetic content.

Lag time
The time taken before resumption of the growth of growth-arrested bacteria.

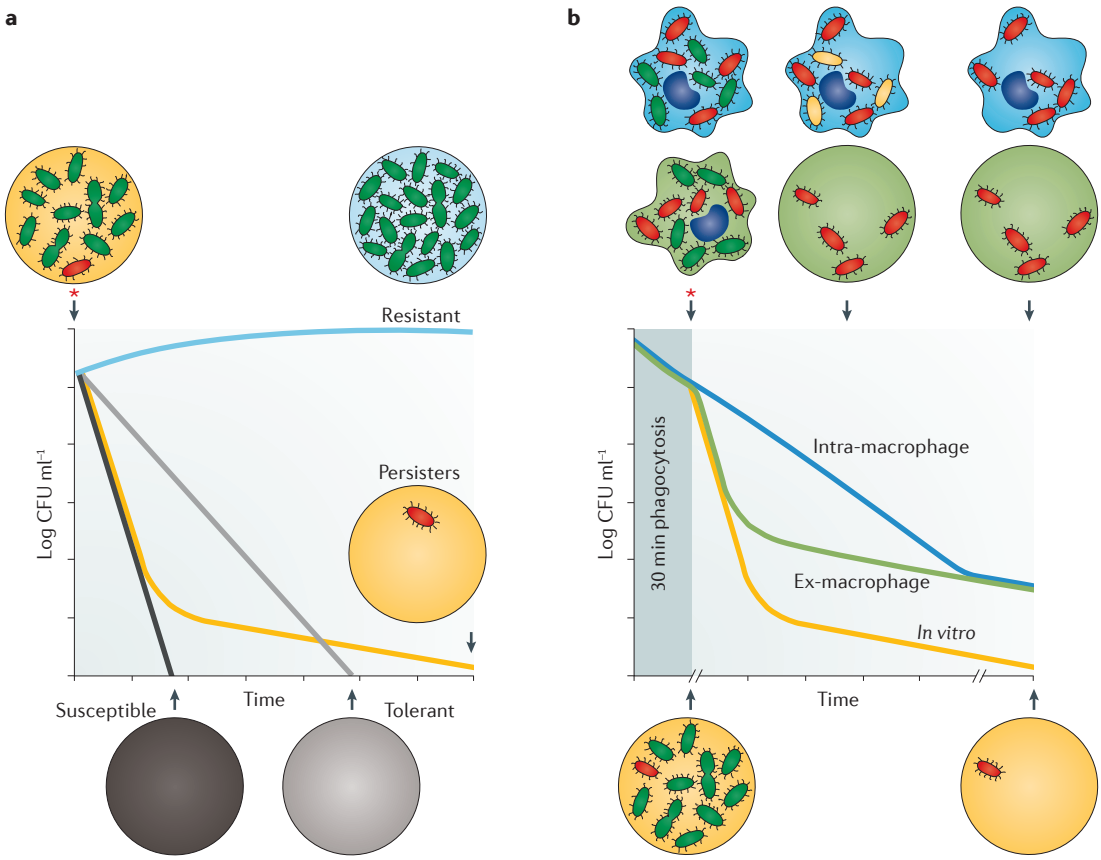

Figure 1 | **Antibiotic killing kinetics of resistant, tolerant and persister cells. a** | Following the addition of a bactericidal antibiotic (red asterisk) to a homogeneous population of resistant bacteria (blue), growth continues. A homogeneously tolerant bacterial population (light grey) takes longer to be killed by an antibiotic than a susceptible population (dark grey). Within a heterogeneous population of clonal bacteria, such as that found in nature, the presence of persisters is revealed by a classic biphasic kill curve (orange) following antibiotic treatment, with a period of rapid killing of susceptible cells followed by a slower decrease in colony forming units (CFU), represented by the persister fraction of cells. **b** | Cefotaxime survival kinetics of *Salmonella* spp. cells grown in Luria-Bertani (LB) medium (orange), *Salmonella* spp. cells grown in LB medium after 30 min of internalization into macrophages (green) and intra-macrophage *Salmonella* spp. cells (blue). The red asterisk indicates the addition of cefotaxime. The *Salmonella* spp. cells that were cultured in LB medium (orange) were mostly cells that are susceptible to killing by antibiotics, represented by the sharp decrease in CFU, with a small persister fraction that can be observed in the subsequent slower decrease in CFU. After 30 min of phagocytosis by macrophages, followed by the release of the *Salmonella* spp. cells into LB medium containing cefotaxime (green), the curve is similar to that of *Salmonella* spp. cells treated with cefotaxime *in vitro*, but with a larger persister fraction. The intra-macrophage *Salmonella* spp. cells (blue) also have a large persister fraction, as evidenced by the slow decrease in CFU towards the end of the curve. The tolerant slow-growing *Salmonella* spp. cells have much slower killing kinetics than the highly susceptible fast-growing *Salmonella* spp. cells in LB medium. Part **b** is adapted with permission from REF. 14, AAAS.

Diauxic shift
A shift in metabolism from one carbon source to another.

Stochastic gene expression
The random (or noisy) fluctuations in the transcription of a particular gene.

Stringent response
A global change in gene expression and protein regulation following amino acid starvation signalled by the alarmones guanosine tetraphosphate and guanosine pentaphosphate (collectively referred to as (p)ppGpp) in bacteria and plants, directing resources away from growth and towards amino acid synthesis to promote survival.

Mechanisms of persister biology

Persister cell formation

Several environmental cues are thought to enhance the formation of persister cells, most of which are various sources of cellular stress, such as nutrient limitation[60], diauxic shift[68], extreme pH[14] and DNA damage[69]. Environments that are inhabited by bacteria during infection, such as within biofilms and macrophages, have also been shown to be associated with the formation of persisters[14,58]. These complex environments are likely to induce the formation of persisters by presenting some, or all, of the cellular stresses described above. The intriguing nature of this phenomenon is that it only occurs in a subset of a clonal population. Why, if an environmental stress is placed on the entire population, does only a subset of the population form persisters? Stochastic gene expression across the population could contribute to phenotypic heterogeneity[70]; however, the formation of microenvironments that cause further stress, such as those that are low in nutrients or high in toxic compounds, could also contribute[60]. The centre of a biofilm is thought to be particularly limited in nutrients, which could explain why only part of the bacterial population forms persisters. This is supported by the requirement for an active stringent response for antibiotic tolerance in biofilms[71]. Macrophages may also restrict nutrient availability and lower the pH to different extents in different cells, or within different phagosomes, thus inducing the

formation of persisters for some bacteria and not for others. However, the exact mechanisms of how these environmental cues induce the formation of persisters are still a matter of debate.

Toxin–antitoxin modules

The link between toxin–antitoxin modules and the formation of persisters first came from a chemical mutagenesis screen that identified a hip mutant of *E. coli* that formed a greater proportion of persister cells than the wild-type strain[52]. The mutation was mapped to the gene *hipA*, which belongs to a toxin–antitoxin module. Around the same time, toxin–antitoxin modules on plasmids were being studied owing to their role in plasmid stability and post-segregational killing[72,73]. It was later realized that many of these toxin–antitoxin modules are also encoded on bacterial chromosomes, and may be involved in inhibiting metabolic processes and reducing growth in response to stress[74,75]. Toxin–antitoxin modules encode a stable toxin that may inhibit any one of a diverse set of metabolic processes and an antitoxin that neutralizes the activity of the toxin when the cell is not under stress. When stressed, the cell may degrade the antitoxin, enabling the toxin to inhibit the activity of its target[76]. Mathematical modelling of the regulation of toxin–antitoxin modules suggests that a bistable state of expression is possible within a clonal population of cells, one that results in the production of many free toxin proteins and another that results in the few toxin proteins that are produced being tightly bound and neutralized by antitoxins[77,78]. This bistability explains how it is possible for a clonal population of cells to contain both growing and growth-arrested cells. Currently, six different types of toxin–antitoxin module have been described, which are categorized by the various mechanisms of neutralization that are used by the antitoxin[79]. The best described types, type I and type II, are implicated in the formation of persisters[14,69,80–83], although this does not exclude the possibility of the other types also being involved. In *E. coli*, the SOS response was found to induce the expression of the type I pore-forming toxin gene *tisB*, thus increasing the formation of persisters in the population[69]. The alarmones guanosine tetraphosphate and guanosine pentaphosphate (collectively referred to as (p)ppGpp) have been linked to the activation of type II and type I toxin–antitoxin modules through signalling cascades that involve Lon protease and the GTPase ObgE respectively, leading to an increase in the formation of persisters[60,83]. Both of these proteins have central roles in bacterial metabolism; Lon protease is involved in the degradation of many cellular proteins[84], whereas ObgE regulates protein translation and DNA metabolism[85]. This discovery suggests an overlap between the regulation of type II and type I toxin–antitoxin modules with central metabolism in bacteria. However, some studies suggest that the role of Lon protease in the formation of persisters in *E. coli* is unclear. The deletion of *lon* in *E. coli* was shown to have no effect on persister levels that were isolated by treatment with β-lactam or aminoglycoside classes of antibiotic[86,87]. The deletion of *lon* did decrease the number of persisters that were isolated by fluoroquinolones; however, the additional deletion of *sulA*, which encodes a cell division inhibitor that is induced by the SOS response, restored persister formation to wild-type levels[86]. As SulA is a substrate of Lon protease[88], the apparent decrease in the number of persisters could be due to unregulated SulA activity inhibiting regrowth after DNA damage, rather than a lack of antitoxin degradation.

Various activities have been described for the toxins currently characterized (FIG. 2). These include inhibiting cellular processes such as translation[89–95], DNA metabolism[96–98] and decreasing the proton motive force that is generated by the electron transport chain[69,83]. Toxins that inhibit protein synthesis seem to be the most widespread, with many targeting RNA, including mRNases (such as RelE[90–92]), tRNases (such as VapC[93,94]) and tRNA-acetylating toxins (such as TacT[99]). There are also several kinases, such as HipA and Doc, that phosphorylate GltX and elongation factor Tu (EF-Tu), respectively, to inhibit their activity[89,95]. Pore-forming toxins, such as TisB and HokB, have been shown to reduce the proton motive force across the bacterial membrane and, consequently, decrease the rate of ATP synthesis in the cell[69,83]. Filamentation induced by cyclic AMP (Fic) toxins produced by *Bartonella schoenbuchensis*, *Yersinia enterocolitica* and *P. aeruginosa* were recently found to block the activity of topoisomerase IV (TopoIV) and DNA gyrase, leading to knotting and concatenation of the bacterial chromosome, which resulted in growth arrest[98].

There have been several studies that have linked toxin–antitoxin modules to the formation of persisters. First, transcriptomics carried out on *E. coli* fractions that were enriched for persister cells showed that the expression of toxin–antitoxin modules was upregulated in these subpopulations of bacteria when compared with growing bacteria[80,81]. Furthermore, it was shown that the deletion of five or more type II toxin–antitoxin modules in *E. coli* led to a decrease in the formation of persisters during exponential growth in rich media[82]. Correspondingly, the overexpression of toxins in bacterial cells was shown to inhibit cell growth and promote an antibiotic-tolerant state that could be countered by the overexpression of cognate antitoxins[80,99–102]. In *E. coli*, the toxin HipA is a serine/threonine kinase that phosphorylates and inhibits the activity of GltX, the glutamyl-tRNA synthetase[89,103], thus decreasing the amount of glutamyl-tRNA in the cell. The shortage of glutamyl-tRNA in the cell triggers an increase in (p)ppGpp that leads to the activation of more toxin–antitoxin modules, which is required for the HipA-induced persister phenotype[104]. Type II toxin–antitoxin modules were also found to be upregulated during macrophage infection by *S.* Typhimurium[14], and the deletion of single toxin–antitoxin modules was shown to lead to a significant decrease in the levels of macrophage-induced persister formation. This may be due to a more stressful environment found within host macrophages than in nutrient-rich media, thus providing a greater trigger for the activation of toxin–antitoxin modules.

Most of the studies of the mechanisms of persister formation have been carried out in *E. coli*, which is a Gram-negative bacterium, and therefore much less is

Post-segregational killing
A mechanism of plasmid maintenance that is used by some low-copy-number plasmids through the action of toxin–antitoxin modules, whereby any progeny bacterial daughter cells produced after division that have not received a copy of the plasmid will be killed through unregulated toxin activity.

SOS response
A global change in gene expression and protein regulation due to the degradation of the LexA repressor caused by the detection of DNA damage in a bacterial cell by the RecA recombinase.

Alarmones
Intracellular signalling molecules that are produced in response to stress.

Proton motive force
The movement of protons down an electrochemical gradient across a membrane to drive ATP synthesis and motility in bacteria.

DNA gyrase
A topoisomerase enzyme that decreases the supercoiling of DNA during replication and transcription through cleaving, rotating and re-ligating the DNA double-strand.

known about how Gram-positive persisters are formed. There is currently little evidence that toxin–antitoxin modules are involved in the formation of persisters in Gram-positive bacteria. Deletion of the toxin–antitoxin modules in *S. aureus* did not affect persister levels[105]. However, overexpression of the toxin $mazF_{SA}$ did induce growth arrest in *S. aureus*, whereas co-expression of the entire $mazEF_{SA}$ module did not affect growth[106]. This suggests that toxin–antitoxin modules in Gram-positive bacteria may function in a similar manner to those in Gram-negative bacteria and are probably involved in stress responses[107]. However, they are perhaps not required for antibiotic tolerance.

The model that describes how the activation of toxin–antitoxin modules through (p)ppGpp alarmone signalling leads to persister formation is an attractive one; however, there is evidence that toxin–antitoxin modules are not the only factors that contribute to the formation of persisters (FIG. 2). The deletion of multiple toxin–antitoxin modules, or the absence of (p)ppGpp, leads to a decreased number of persisters, but neither fully abrogates the presence of persister cells[60,82,108]. It is possible that there is heterogeneity within the persister population, with several different pathways contributing to the formation of persister cells. A screen of *E. coli* single-gene deletion mutants from the Keio collection — a collection of deletion mutants in *E. coli* that covers all nonessential genes[109] — did not find any single-gene deletions that could prevent the formation of persisters entirely; however, the screen identified several global regulators that are important for the phenomenon[110]. An alternative model proposes that metabolic regulation is the main driver of persister formation and that toxin–antitoxin modules are only accessory to this control by locking the growth arrest[13,111]. One study recently proposed this was the case for ampicillin-tolerant bacteria that are formed through a diauxic shift between the carbon sources glucose and fumarate[112]. In addition, two different pathways, both of which involve *trans*-translation and the stringent response, have been described as important for the tolerance of persisters formed in response to a diauxic shift and isolated by either β-lactams or fluoroquinolones[113]. However, exactly how these pathways lead to tolerance is currently unknown. Two studies recently described, using either β-lactams or fluoroquinolones, that the formation of persisters in *E. coli* and *S. aureus* is associated with a decrease in ATP levels[105,114], without an obvious dependency on toxin–antitoxin modules. Further investigation is required to determine the exact roles of central metabolism and toxin–antitoxin modules in the formation of persisters.

Phenotypic resistance mechanisms

Phenotypic resistance (BOX 1) enables a subpopulation of bacteria to grow in the presence of a drug, owing to a non-heritable change that enables the degradation, cellular export or chemical modification of an antibiotic, or, alternatively, the modification or downregulation of a drug target. This is in contrast to persister cells, which become tolerant to antibiotics through target inactivity as a consequence of a decrease in growth and metabolism, or through reduced drug uptake. An example of phenotypic resistance is that of the survival and growth of a subpopulation of *M. tuberculosis* during zebrafish infection that was found to be dependent on efflux pump activity[11]. A recent study also described increased levels of the efflux pump component TolC in a proportion of an *E. coli* population that survived killing after treatment with a β-lactam antibiotic. The deletion of *tolC* led to a decrease in survival levels[115]. Another example of phenotypic resistance was described in *M. tuberculosis*, whereby a small proportion of *M. tuberculosis* cells were found to escape killing by isoniazid and grow in the presence of the drug. Isoniazid is a pro-drug that has to be converted into its active form[12] by the bacterial enzyme

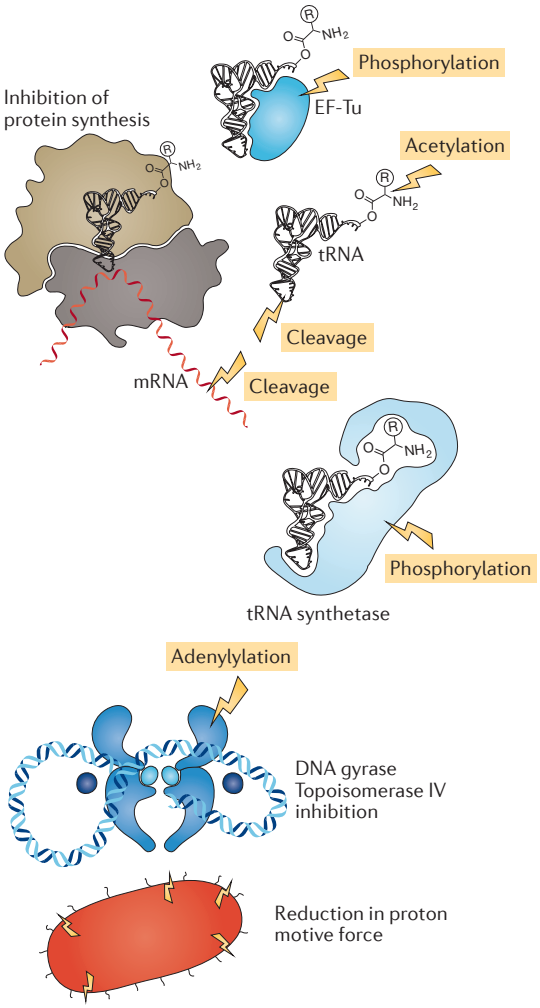

Figure 2 | **Mechanisms of persister formation.** Toxin–antitoxin-mediated mechanisms of persister formation include the inhibition of protein synthesis, DNA metabolism and proton motive force. Protein synthesis is inhibited by various toxins through the phosphorylation of elongation factor Tu (EF-Tu) by Doc[95], the acetylation of aminoacyl-tRNA by TacT[99], the phosphorylation of GltX by HipA[89], and the cleavage of mRNA by RelE[90] or tRNA by VapC[93]. The activity of DNA gyrase and topoisomerase IV (TopoIV) is inhibited by Fic[98] through adenylylation, and pore-forming toxins, such as TisB, are able to reduce the proton motive force of the bacterial cell[138].

Trans-translation
A quality control mechanism in protein synthesis that uses transfer-messenger RNA (tmRNA) to rescue a ribosome that has stalled during translation.

Isoniazid
An antibiotic that is commonly used for the treatment of tuberculosis. Isoniazid is a pro-drug that is converted into its active form by the catalase enzyme KatG inside bacterial cells, it is then able to inhibit the cytochrome P450 system, leading to the production of lethal free radicals.

Box 3 | **Current methods for eradicating persisters**

Persisters escape killing by antibiotics owing to lower metabolic flux, target inactivity, and low uptake or high efflux of antibiotics. Using antibiotics that do not require a metabolically active target to kill bacteria is one strategy that is being investigated. Several small molecules and antimicrobial peptides (AMPs) have been described that target the bacterial cell membrane to kill persisters[139–142]. The acyldepsipeptide ADEP4 was found to activate the ClpP protease in *Staphylococcus aureus* persister cells, thus leading to the deregulated degradation of many proteins, resulting in cell death[61]. Another strategy that is being used to kill persisters is re-sensitizing persisters to traditional antibiotics by forcing them to reinitiate growth or increase their metabolic activity. Saccharides have been used to increase the proton motive force in *Escherichia coli* and *S. aureus* persisters, thus increasing the uptake of aminoglycoside antibiotics[123]. In addition, brominated furonones have been shown to sensitize *Pseudomonas aeruginosa* persisters to antibiotic treatment, although the mechanisms that underlie this observation are currently unknown[124]. Chemical modification of antibiotics is also producing promising anti-persister candidates. Pentobra is a tobramycin molecule that has been peptidylated to aid entry into bacteria, in which it inhibits translation[143]. An antibody–antibiotic conjugate was designed to target *S. aureus*, thus opsonizing extracellular bacteria and promoting phagocytosis. The conjugated drug is only active and kills bacteria inside the phagolysosome. This treatment was used to prevent the cell-to-cell transfer of bacteria during infection[144]. Bacteriophage endolysins are able to lyse Gram-positive bacteria and have been fused with outer membrane-destabilizing peptides to promote the efficient killing of Gram-negative persisters[145,146].

The increase in the number of anti-persister treatments is encouraging; however, there is little translation from the laboratory to the clinical setting. This is because of the large investment of time and money that is required for drug development. Moreover, toxicity to the host often prevents a new antibiotic from being used in patients in the clinic. A greater focus on using cell culture and *in vivo* models in the laboratory to test new anti-persister molecules will increase the likelihood of identifying non-toxic drugs that are more attractive candidates for development.

KatG before it can kill the cell. Some bacterial cells were shown to express lower levels of KatG than the rest of the population. Thus, the levels of the active form of the antibiotic are decreased owing to the downregulation of a specific enzyme, which is indicative of an antibiotic resistance mechanism. Furthermore, a subpopulation of *Enterobacter cloacae* that is phenotypically resistant to the antibiotic colistin was recently reported[116]. Colistin resistance was dependent on PhoQ — the sensor in the PhoPQ two-component system. Interestingly, the proportion of the population that showed resistance to the drug increased within macrophages. The study described one particular strain of *E. cloacae* that formed an antibiotic-resistant subpopulation that was determined to be susceptible to the antibiotic in laboratory tests commonly used in the clinic, whereas the treatment of an infection caused by this strain *in vivo* would probably fail because the resistant subpopulation of bacteria would survive and grow. This highlights the need for routine clinical tests that take into account phenotypic heterogeneity when testing for antibiotic resistance. A further example of phenotypic resistance was recently described in *S. aureus*[117], in which a novel daptomycin resistance mechanism was found whereby *S. aureus* produces lipids that sequester the antibiotic. However, the secretion of toxins known as phenol-soluble modulins, which are under the control of the accessory gene regulator (Agr) quorum sensing system[118], interferes with this resistance mechanism, thus maintaining bacterial susceptibility to the drug. Interestingly, host signals, such as apolipoprotein B (*APOB*), found in human serum are known to inhibit the expression of Agr[119], which could give rise to phenotypic resistance to daptomycin during infection. Many of these examples act as a reminder that antibiotic treatment failure may be due to specific interactions between the drug, pathogen and host, and may be difficult to understand using *in vitro* testing alone.

Regrowth of persisters

Molecular mechanisms of regrowth. How growth-arrested persisters are able to switch back to a growing state remains an important unanswered question in persister biology. Understanding the mechanisms behind this process is of substantial importance because reinitiating growth — and thereby increasing drug susceptibility — may be an effective method of killing persisters when combined with antibiotics (BOX 3). Persisters have been observed either regrowing all together[112] or spontaneously[51] as soon as an external stress is removed[112]. These differences in patterns of regrowth are due to the type of persister that is being studied under specific experimental conditions and the levels of heterogeneity within the persister subpopulation.

It is known that the activation of toxin–antitoxin modules can lead to the formation of persisters in Gram-negative bacteria; however, how this activation can be reversed remains unknown (FIG. 3). Several type II toxin–antitoxin modules have been shown to autoregulate their expression as a consequence of conditional cooperativity[79], which enables the repression of the toxin–antitoxin operon when toxin:antitoxin ratios are low and derepression when free toxin levels are high[120]. Presumably, if antitoxin degradation stops, perhaps when a stress signal is reduced, then the toxin:antitoxin ratio may return to a level that enables repression of the operon. Proteolytic degradation of the toxin could also occur. Moreover, as the antitoxin gene is typically found upstream of the toxin gene, it is possible that abortive transcription may also be involved in the determination of toxin:antitoxin ratios. It is unclear exactly how the cellular pool of antitoxin is replenished when the toxin inhibits translation; however, mathematical modelling of conditional cooperativity has predicted this to be possible[78], given that a small amount of translation is allowed to continue. However, experimental evidence that demonstrates a transition from derepression to repression is currently lacking. It is important to consider that some toxin–antitoxin modules are not regulated through conditional cooperativity and therefore other regulatory mechanisms must exist in these cases[79].

The repression of toxin expression might be insufficient to enable bacteria to return to a growing state, and in some cases detoxification might also be required to reverse the effects of the toxin[121]. The toxins CcdB and ParE preferentially bind to their cognate antitoxins over their target (DNA gyrase)[96,97], thus titrating out any active toxin. The toxicity that is induced by RelE and MazF is reversed by transfer-messenger RNA (tmRNA), which rescues ribosomes after stalling on mRNA cleaved by the toxins[91,122]. However, many toxins inhibit their targets through the addition of post-translational modifications. It was recently shown that TacT in *S.* Typhimurium

Antimicrobial peptides
(AMPs). Small peptides synthesized by plants and animals that have antimicrobial properties and often target the bacterial membrane.

PhoPQ two-component system
Two-component systems comprise a sensor and a regulator that are able to detect environmental changes and mediate transcriptional changes in response. The PhoPQ system regulates the glycerophospholipid and protein content of the outer membrane in response to pH.

Conditional cooperativity
The autoregulation of certain toxin–antitoxin modules, whereby the antitoxin and toxin are able to form a complex only at a certain stoichiometric ratio, which can then effectively repress transcription of the module.

blocks translation through the acetylation of aminoacylated tRNA molecules[99], thus inhibiting growth. However, this inhibition can be overcome by overexpressing Pth, which is a peptidyl tRNA hydrolase that is reported to cleave peptide chains off tRNA molecules after ribosome stalling[114,115]. Pth is able to recognize and cleave acetylated amino acids from tRNA molecules that have been modified by TacT, which enables the recycling of tRNA. Experimental evidence is currently lacking for other detoxification mechanisms; nevertheless, it is possible to speculate as to what these mechanisms might involve on the basis of the activity of the toxin. Toxins that add post-translational modifications to their targets may have their modifications removed by other cellular enzymes. For example, the dephosphorylation of EF-Tu and GltX could reverse the toxicity of Doc and HipA, respectively, and the deampylation of TopoIV and DNA gyrase could reverse Fic toxicity.

Environmental restriction of growth. The possible mechanisms that enable persister regrowth that are described above are internal factors; however, bacteria also require an unrestrictive environment to grow. External factors that determine regrowth could include chemical cues, relief of physical stresses or the presence of an essential nutrient (FIG. 3). Small molecules have been shown to stimulate metabolism in *E. coli* and *P. aeruginosa* persisters[123,124] (BOX 3); however, as they do not fully restore the ability to proliferate, it is hypothesized that there are unknown factors that prevent regrowth. Determining the underlying mechanism of action of these molecules and the cellular factors that are responsible for this growth block could reveal important insights into persister biology. In the case of host immune responses, it has been known for a long time that suppression of the immune system can lead to the relapse of latent infections[125]. If IFNγ-mediated immune responses are downregulated in epithelial cells, then intracellular *Chlamydia trachomatis* persisters are able to resume growth[126]. This is due to the relief of the restriction that is imposed on bacterial growth by innate defences and nutrient limitation when inside activated immune cells. This is particularly important for individuals who are immunosuppressed. In addition, persisters can regrow after infecting a different host cell[14]. Theoretically, host cells that contain persisters *in vivo* will eventually die and release these bacterial cells. After the downregulation of an IFNγ-mediated immune response, a newly acquired host cell may be more permissive to bacterial growth. If immune responses are a major factor for blocking the regrowth of persisters, it may be possible to induce bacterial regrowth, and thus re-sensitize persisters to drugs by modulating host immunity. However, as this could involve suppressing the immune system, it may not be an option for some patients.

State of regrowers. What condition will persister cells be in once regrowth has initiated? It was suggested that because the persister state is strongly linked to the stress response, regrowing persisters may be primed for mutation and adaptation[6]. It is possible that regrowing persisters express higher levels of proteins that are involved in DNA repair, such as error-prone polymerases, as these are associated with the SOS and stress responses[127,128]. The expression of error-prone polymerases could increase the rate of mutation during replication, thus theoretically increasing the probability of the emergence of antibiotic resistance during persistent infections, particularly if low levels of the drug are still present in the host. The SOS response, which has been shown to induce the formation of persisters[69,129], is also known to upregulate mechanisms of horizontal gene transfer, such as conjugation and phage activation[130]. This could promote the spread of antibiotic resistance following persister regrowth. One recent study showed that DNA repair mechanisms and the SOS response are important for persister regrowth after treatment with ofloxacin[131]; however, whether these responses are a general feature of persisters isolated with other classes of antibiotic that do not target DNA specifically remains unknown. Combining evolution experiments with the close monitoring of antibiotic tolerance and resistance in populations of *E. coli* found that mutations that confer resistance to ampicillin after sequential treatments were preceded by mutations that confer an increase in antibiotic tolerance. This suggests that tolerant bacteria and persister cells are an important bacterial reservoir for the emergence of antibiotic resistance[7].

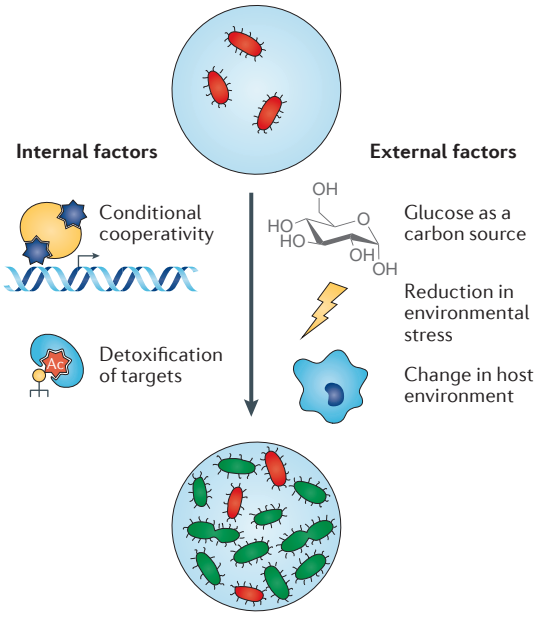

Figure 3 | **Mechanisms of persister regrowth.** Internal and external factors determine the ability of persisters to regrow. Internal factors include the repression of toxin–antitoxin modules that are regulated through conditional cooperativity by lowering toxin:antitoxin ratios[78]. Post-translational modifications (PTMs) that were added to cellular substrates by toxins will have to be removed to recycle cellular components, as is the case for TacT and Pth[99]. External factors include the addition of a previously limited nutrient, such as glucose[112], or the reduction or removal of a limiting environmental stress. A change in host cell has been shown to enable regrowth[14].

Transfer-messenger RNA
(tmRNA). A specialized form of RNA that mimics a tRNA with an mRNA-like coding element that is used during *trans*-translation to continue stalled protein synthesis and target the resultant aberrant protein for degradation.

Deampylation
The removal of an adenylyl group.

Conclusions

Persistent infections cause high levels of morbidity and mortality globally. Persisters are transiently antibiotic-tolerant cells that are thought to be ubiquitous among bacteria and have been linked to the relapse and recalcitrance of persistent bacterial infections, and probably aid the spread of antibiotic resistance. Owing to an increase in the number of immunocompromised patients and the spread of antibiotic resistance, these recurrent and recalcitrant infections are likely to become more prevalent in the future. The persister phenomenon is also associated with fungal infections[55] and even the treatment of malignant tumours, in which some growth-arrested tumour cells are tolerant to anticancer drugs[132]. A greater understanding of the internal and environmental factors that lead to the formation of persisters and their regrowth will enable the development of improved treatments for persistent infections.

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The authors declare no competing interests.