Persister cells, dormancy and infectious disease

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Abstract | Several well-recognized puzzles in microbiology have remained unsolved for decades. These include latent bacterial infections, unculturable microorganisms, persister cells and biofilm multidrug tolerance. Accumulating evidence suggests that these seemingly disparate phenomena result from the ability of bacteria to enter into a dormant (non-dividing) state. The molecular mechanisms that underlie the formation of dormant persister cells are now being unravelled and are the focus of this Review.

Dormant
A dormant cell has a global slowdown of metabolic processes and does not divide.

Tolerance
The ability of cells to survive killing by antibiotics without expressing or using resistance mechanisms.

Non-proliferation
A cell that does not divide.

Chaperone
A protein that mediates the assembly of another polypeptide-containing structure, but does not form part of the completed structure, or participate in its biological function.

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Persisters were described by Joseph Bigger in 1944 in one of the first studies on the mechanism of penicillin action[1]. Bigger discovered that penicillin lysed a growing population of *Staphylococcus* spp., but that a small number of persister cells, which were not simply antibiotic-resistant mutants, inevitably survived (FIG. 1a) and proposed that these were dormant, non-dividing cells. Harris Moyed continued working on the problem in the 1980s and carried out a targeted search for persister genes[2–6]. He treated a population of *Escherichia coli* with ampicillin, then allowed the surviving cells to grow in the absence of antibiotic. After several such cycles, two colony types were identified following plating on solid media; these were conventional antibiotic-resistant mutants that grew in the presence of ampicillin, and mutants that produced persister cells at a higher frequency, but that were unable to grow in the presence of ampicillin.

One of these persister mutations was mapped to the *hipA* gene. The *hipA7* allelic strain produced ~1% persisters that survived treatment with ampicillin in exponential cultures, which is approximately 1,000 times more persisters than the wild-type strain.

The recent discovery of persisters in biofilms[7,8] has rekindled interest in these unusual cells. Indeed, the presence of persister cells might be important in the aetiology of many recalcitrant infectious diseases. In this Review, I will summarize the biology of persister cells and offer insights into their role in infectious disease.

Multidrug resistance and multidrug tolerance

Numerous mechanisms of drug resistance have been described, and in most cases we have a good understanding of these processes at the molecular level. The main types of resistance are target modification by mutation; target modification by specialized enzymatic changes; target substitution, such as expressing an alternative target; antibiotic modification or destruction; antibiotic efflux; and restricted permeability to antibiotics[9,10]. It is interesting to note that all of the theoretically logical possibilities of antibiotic resistance seem to have been realized in nature. Importantly, all of these mechanisms accomplish the same aim, which is to prevent the antibiotic from binding to its target (FIG. 2). Each of these resistance mechanisms allows cells to grow at an elevated concentration of antibiotic. Bactericidal antibiotics kill the cell not by inhibiting the target, but by corrupting its function to create a toxic product. Aminoglycoside antibiotics kill the cell by interrupting translation, which produces misfolded toxic peptides[11]. Beta-lactam antibiotics, such as penicillin, inhibit peptidoglycan synthesis, which activates, by an unknown mechanism, autolysin enzymes present in the cell wall[12]. This leads to digestion of the peptidoglycan by autolysins and cell death. Fluoroquinolones inhibit the ligase step of the DNA gyrase and topoisomerase, without affecting the preceding nicking activity. As a result, the enzymes are converted into endonucleases[13].

In contrast to resistance, the tolerance of persisters to antibiotics might function by preventing target corruption by a bactericidal agent through the blocking of the antibiotic target(s) (FIG. 2). If persisters are dormant and have little or no cell-wall synthesis, translation or topoisomerase activity, then the antibiotics will bind to, but will be unable to corrupt, the function of their target molecules. In this way tolerance could enable resistance to killing by antibiotics, but at the price of non-proliferation.

Production of persister cells

The simplest route to form a dormant persister cell might be through the overproduction of proteins that are toxic to the cell and inhibit growth. Recombinant *E. coli* cells that overproduce the chaperone DnaJ, or the PmrC
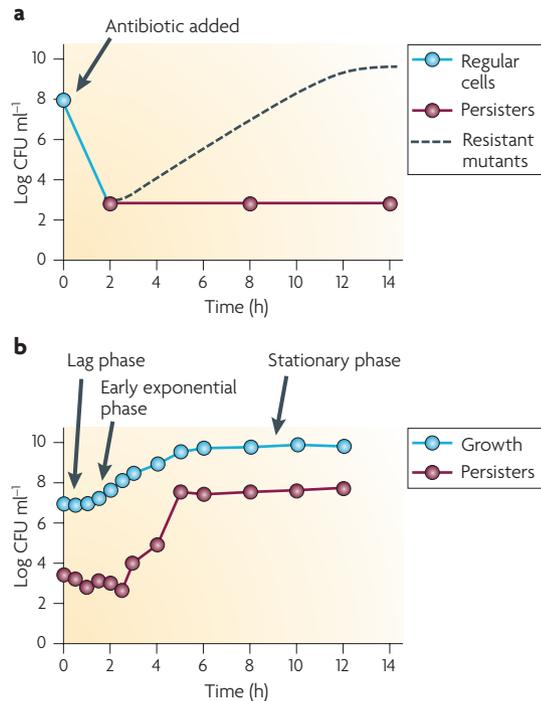
Figure 1 | **Formation of persister cells. a** | Shows the treatment of a population with an antibiotic, which results in cell death, leaving only persister cells or resistant mutants alive. **b** | Shows the frequency of isolation of persisters as a function of the growth phase of the culture.

protein from *Salmonella entericia* serovar Typhimurium (*S. typhimurium*), stopped growing and became highly tolerant to ampicillin and ciprofloxacin[14].

However, this simple mechanism is unlikely to be responsible for the natural production of persisters. Examination of the rate of *E. coli* persister-cell formation over time showed that few of these cells are formed in early exponential phase, followed by a sharp increase in persister-cell formation in mid-exponential phase, reaching a maximum of ~1% of cells forming persisters in the non-growing stationary phase (FIG. 1b). In order to probe whether persisters isolated during the early exponential phase of growth were introduced from a stationary state culture inoculum, or were formed *de novo*, the culture was kept in early exponential phase by repeated dilution and regrowth[15]. After four cycles, persister cells completely disappeared. This simple experiment rules out non-specific mechanisms of persister formation and indicates that persister cells are preformed, rather than being produced in response to antibiotics.

The formation of persisters rapidly increases during mid-exponential phase in several species, but the mechanism that underlies this remains a puzzle. Quorum sensing does not seem to have a role in persister formation, because the addition of spent growth medium (which would contain high concentrations of quorum-sensing signalling molecules) to early exponential cultures of *E. coli* or *Pseudomonas aeruginosa* did not appreciably increase the number of persisters isolated (K. L.,

unpublished observations). Whatever the mechanism, the dynamics of persister formation provide an interesting insight into this strategy of persistence. Because the highest frequency of persister formation occurs in the stationary phase of growth their main function might be to ensure the survival of this non-growing population. As persisters do not grow, it would seem logical for the entire stationary population to enter into this protected survival state, but this is not the case. The benefit of being a regular, dividing cell seems to stem from its ability to rapidly resume growth, a process that takes 1–1.5 hours longer for a dormant persister cell[16]. Because most cells in an *E. coli* or *P. aeruginosa* stationary phase population are non-persisters, this indicates that the optimal individual strategy is not to enter into persistence, suggesting that the persister state is an altruistic behaviour benefiting the kin. Indeed, in the rare instance that the whole population of normal cells is killed by a lethal factor, the surviving persisters can propagate the genome that they share with their kin (BOX 1).

Genetics of persister formation
Attempts to identify genes that are required for persister-cell formation by transposon mutagenesis were unsuccessful[17,18]. Such experiments are problematic owing to the variation in the numbers of persisters identified in parallel replicates[19], which leads to an unmanageable level of false-positives and false-negatives when screening a large library. An alternative method is to identify genes by gain-of-function from an expression library. However, overproduction of some proteins can result in toxic effects that restrict cell growth, which results in the isolation of false-positives. It seems that standard genetic approaches are poorly suited to searching for persister genes.
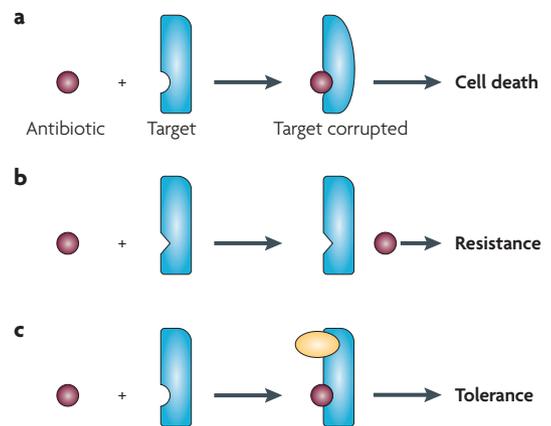
Figure 2 | **Resistance versus tolerance to bactericidal antibiotics. a** | The antibiotic (pink) binds to the target (blue) altering its function, which causes cell death. **b** | The target of the antibiotic has been altered so that it fails to bind the antibiotic and the cell becomes resistant to treatment with the drug. **c** | A different molecule (yellow) inhibits the antibiotic target. This prevents the antibiotic from corrupting its functions, resulting in tolerance. CFU, colony forming unit.

Quorum sensing
The ability of bacteria to sense their own cell density by detecting the concentration of signalling molecules that have been released in their environment.

Signalling molecule
A chemical, similar to a pheromone, that is produced by an individual bacterium. Signalling molecules can affect the behaviour of surrounding bacteria.

Box 1 | **Unculturable bacteria**

Over 99% of all species that are present in the environment fail to grow on laboratory media[73]. Recent research provides us with some important insights into the problem. A general method to grow unculturable bacteria from various environments has been described[74]. The basic approach is to grow cells in their natural environment. Cells are taken from an environment, such as marine sediment, diluted, mixed with agar and sandwiched between two semi-permeable membranes of a diffusion chamber. The chamber is then placed back in the same environment that the sample originated from, such as the sediment surface of a marine aquarium. The chamber allows the free diffusion of nutrients and other substances, while excluding cells. After incubation, colonies of bacteria grow in the chamber, and most environmental organisms can be recovered and isolated. Similarly, placing a sample originally taken from the water column back into marine water resulted in the cultivation and sequencing of *Pelagibacter ubique*, the first representative of the unculturable bacterial domain SAR11 — one of the most common marine bacteria — that was previously known only by its 16S signature[75–78].

We have observed that unculturable organisms will readily grow on nutrient medium on a Petri dish *in vitro* in the presence of other species from the same environment[74]. This observation might indicate that growth depends on substances (perhaps signalling molecules) that indicate the presence of a familiar environment. Most bacterial species have evolved to grow in a specific environment and fail to propagate when placed in a synthetic medium. A cell dividing in an unfamiliar, but nutrient-rich environment exposes itself to an uncertain fate of being killed by an unfamiliar antibiotic to which it has no resistance. It is tempting to hypothesize that unculturable cells enter into a dormant state that is similar to persisters when transferred to an unfamiliar environment. Even a simple lack of growth is a partial state of dormancy that might be protective. Many well-characterized bacteria, such as *Escherichia coli*, can propagate on different laboratory growth media. By contrast, unculturable bacteria do not grow in perfectly nutritious media, but require specific components that mimic their usual environmental niche. From this perspective, dormancy might be the default form of most bacterial life.

Another barrier to the discovery of persister genes has been a lack of methods for the isolation of persister cells. The first method to isolate persisters was recently reported, based on the simple sedimentation of surviving cells from a culture that had been lysed by treatment with ampicillin[20]. Expression profiling of RNA from isolated persister cells revealed the downregulation of transcription of genes involved in energy production and non-essential functions such as flagellar synthesis, which might indicate that persisters are dormant, consistent with their phenotype of slow or non-growth[16]. Specific genes were identified that might contribute to the dormant phenotype; *rmf*, a stationary state inhibitor of translation[21], *sulA*, an inhibitor of septation[22], and toxin–antitoxin (TA) loci *relBE*, *dinJ* and *mazEF*[23,24]. Homologues of TA modules are found on plasmids in which they constitute a maintenance mechanism[25]. Typically, the toxin is a protein that inhibits an important cellular function such as translation or replication, and forms an inactive complex with the antitoxin. The toxin is stable, whereas the antitoxin is degradable. If a daughter cell does not receive a plasmid after segregation, the antitoxin level decreases owing to proteolysis, leaving a toxin that either kills the cell or inhibits propagation. TA modules are also commonly found on bacterial chromosomes, but their role is largely unknown[20,26]. MazEF was proposed to function as a programmed cell-death mechanism[27]. However, it was subsequently reported that MazF and the unrelated toxin RelE do not actually kill cells, but instead induce cell stasis by inhibiting translation, and that this state can be reversed by expression of the cognate antitoxins[23,28].

Plasmid-mediated overexpression of RelE or MazF in *E. coli* strongly increased tolerance to antibiotics[14,20]. Expression of the toxin HipA also increased antibiotic tolerance[14,29–31]. Interestingly, bioinformatics indicate that HipA is a member of the phosphoinositol 3/4-kinase superfamily, which has been extensively studied in eukaryotes[32], but has not previously been identified in bacteria. HipA was found to autophosphorylate on Ser150, and site-directed mutagenesis replacing this residue, or other conserved amino acids in the catalytic and Mg^{2+}-binding sites, abolished the ability of HipA to stop cell growth and confer drug tolerance[31]. The target of the HipA kinase has yet to be identified. Deletion of *rmf*, *relE* or *mazF* had no discernible persister-production phenotype, possibly owing to the redundancy of these elements — in *E. coli* there are at least ten TA modules[33].

Several independent lines of evidence indicate the dormancy of persisters — lack of growth in the presence of antibiotics, downregulation of biosynthetic pathways, and an elegant demonstration of slow or no growth of persisters formed by the *E. coli hipA7* strain[16].

Based on these data, we reasoned that dormancy could be used to physically sort naive persister cells (those that have not been exposed to antibiotics) from the population[34]. Dormant cells have low levels of translation, which can enable differential cell sorting based on the expression of a detectable protein. In *E. coli* strain ASV, a green fluorescent protein (GFP) reporter gene that encodes a degradable protein was inserted into the chromosome downstream of the ribosomal RNA gene promoter, *rrnBP1*. The transcriptional output of the *rrnBP1* promoter is proportional to the rate of cell growth (FIG. 3). Sorting of dim cells — in which the amount of GFP that is produced is low, indicating little or no transcription from the *rrnBP1* promoter — from the population led to the isolation of antibiotic-tolerant persisters. However, a rapid method for obtaining large numbers of persisters has yet to be developed.

A generalized hunt for persister genes. Moderate overexpression of a genomic library (constructed in plasmid pACYC184) enabled the identification of *E. coli* genes that, on overexpression, increased the frequency of persister production[18]. One of the genes that led to increased production of persisters on overexpression was the glycerol-3-phosphate (G3P) dehydrogenase
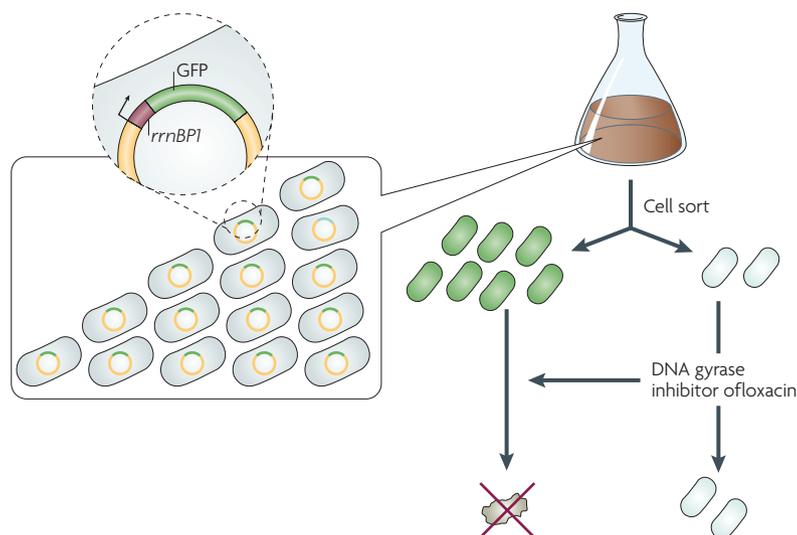
Figure 3 | **A method for isolating persisters.** *Escherichia coli* strain ASV cells containing a plasmid expressing the *rrnBP1* promoter fused to a degradable green fluorescent protein (*gfp*) reporter gene are grown to mid-exponential phase then sorted with a high speed cell-sorter that is equipped with a standard GFP filter set. Normally growing bright (fluorescent) cells are killed by ofloxacin. However, cells that have low levels of GFP translation, and are dim, are tolerant to ofloxacin, indicating that they are persisters.

gene (*glpD*). Deletion of the *glpD* locus from a wild-type *E. coli* strain reduced the production of persisters in a stationary (but not exponential) phase culture. However, how the expression of *glpD* affects persister formation is unclear. Testing mutants that were mutated in genes in the *glpD* metabolic network led to the discovery of another interesting gene that affects the formation of persisters — the gene encoding PlsB, the (G3P) acyl-transferase. The PlsB enzyme catalyses the first step in phospholipid synthesis, using G3P to produce 1-acyl-G3P. The *plsB* gene is essential, which precludes the construction and analysis of a null mutant. However, an *E. coli* strain that synthesizes a mutant form of the PlsB enzyme that has an increased Michaelis constant (K_m) compared with the wild-type enzyme has been described (*plsB26*) (REF. 35), and was used to examine persister formation. The *plsB26* strain grew normally and had the same minimum inhibitory concentration (MIC) for ampicillin and ofloxacin as an isogenic wild-type control. Production of persisters by the *plsB26* strain in a stationary culture was 100–1000 times lower than the wild-type strain, indicating that PlsB might be a useful target for anti-persister therapy. PlsB is constitutively expressed, and the level of *plsB* mRNA in persisters was not altered compared with the amount found in wild-type cells[20,34]. It seems likely that PlsB is not a persister protein that is required for the formation of dormant cells, but rather is essential for maintenance of the persistent state.

The plethora of proteins that can potentially induce multidrug tolerance is reminiscent of the many multidrug pumps responsible for multidrug resistance (MDR). For example, in *P. aeruginosa* there are genes that encode 15 MDR pumps belonging to the

resistance-nodulation-cell division (RND) family alone, of which only one pump, MexAb–OprM, is expressed at a high level under laboratory conditions[36]. Knockouts of most *mdr* genes have no phenotype, whereas over-expression of *mdr* genes produces a functional MDR pump[37]. It seems that microbial populations have evolved two complementary and redundant strategies to protect themselves against antimicrobials: multidrug efflux mechanisms, and if this fails to protect the cells, the multidrug tolerance of persister cells can preserve the population.

Persisters and stochastic phenomena. Persisters comprise a small sub-population, and in mid-exponential phase *E. coli* produces as few as 10^{-5} surviving antibiotic-tolerant persister cells. Given that all of the cells in a population are genetically identical it seems that persisters must be produced by a stochastic process[20,38,39]. Indeed, what is the alternative? Fluctuations in the concentrations of a small number of dedicated proteins are probably responsible for the formation of persisters. The absence of persisters in an early exponential population might be due to low concentrations of persister proteins at this stage. It seems that two processes control persister formation — a stochastic fluctuation in the level of specific proteins (persister proteins), and a controlled, regulated mean level of expression of these proteins, which is dependent on the density of the population, and probably on several other factors as well[15]. The environment therefore sets the baseline over which stochastic fluctuation of expression will take place.

Stochastic processes have been described for a large number of functions, both in bacteria[40,41] and in eukaryotes[42]. In bacteria, stochastic processes are responsible for determining the members of the population that enter sporulation in *B. subtilis*[43]; spontaneous SOS induction in *E. coli*[44]; or cannibalism in *B. subtilis*[45]. The most visible case of bacterial decision-making is chemotaxis, which relies on a trial-and-error random walk[46]. Two particular proteins, CheZ and CheY, of the many elements involved in the chemotaxis signal transduction seem to be responsible for the noise generation that determines whether a cell will run or tumble, which in turn determines when a cell changes direction[47]. The reason bacteria use stochastic processes in decision-making is probably similar to flipping a coin when a problem does not have a unique logical solution. Therefore, when a population needs to produce a small number of specialized survivor cells, fluctuations in the level of persister proteins will induce a dormant state in a random set of cells.

Interestingly, stochastic processes seem to contradict the well-established evolutionary paradigm of evolving to perfection. Perfection in regulatory functions is driven by economy, proteins are produced as needed and at optimally required levels. Perfectly regulated expression has no noise and will produce a population of identical cells. In processes controlled by stochasticity, evolution to perfection would have been maladaptive. Settling for a less than perfect level of control results in useful variety.

Chemotaxis
The movement of bacteria towards nutrients and away from toxins.
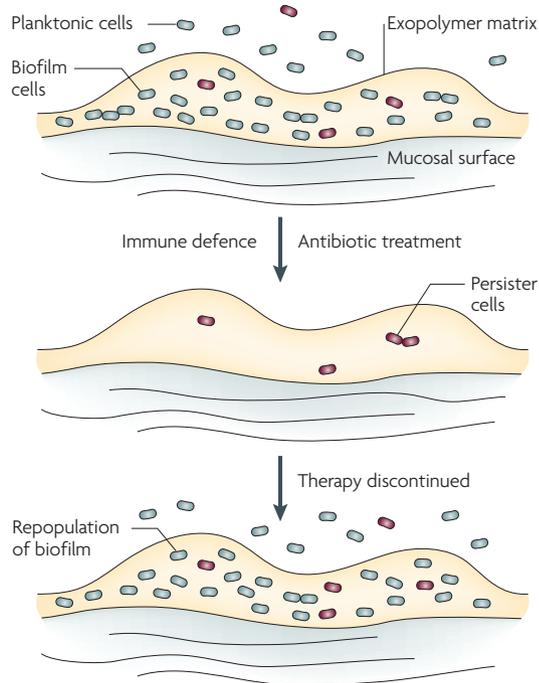
Figure 4 | **Biofilm drug resistance.** The figure shows a model of biofilm resistance to killing based on persister survival. Initial treatment with antibiotic kills normal cells (coloured green) in both planktonic and biofilm populations. The immune system kills planktonic persisters (coloured pink), but the biofilm persister cells (coloured pink) are protected from the host defences by the exopolymer matrix. After the antibiotic concentration is reduced, persisters resuscitate and repopulate the biofilm and the infection relapses. Modified with permission from REF 51 © (2001) American Society for Microbiology.

Biofilms and persisters

According to the CDC, 65% of all infections in developed countries are caused by biofilms, which are bacterial communities that settle and proliferate on surfaces and are covered by an exopolymer matrix[48]. Infections ascribed to biofilms include: common diseases such as childhood middle-ear infection and gingivitis; infections of all known indwelling devices such as catheters, orthopaedic prostheses and heart valves; and biofilm infections also occur in sufferers of incurable cystic fibrosis. Biofilms can be formed by most, if not all, pathogens. *P. aeruginosa*, which causes an incurable infection in cystic fibrosis patients[49] and *Staphylococcus aureus* and *Staphylococcus epidermidis*, which cause infections of indwelling devices[50], are probably the best-known biofilm-producing organisms. Biofilm infections are highly recalcitrant to antibiotic treatment. However, planktonic cells that are derived from these biofilms are, in most cases, fully susceptible to antibiotics. Importantly, biofilms do not actually grow in the presence of elevated concentrations of antibiotics, therefore biofilms do not have increased resistance compared with planktonic cells[51]. But if biofilms are not resistant to antibiotics, how do biofilm bacteria avoid being killed by antibiotic treatments? The resistance

of biofilms to drug therapy has been one of the more elusive problems in microbiology, but the analysis of a simple dose-response experiment provided an unexpected insight into the puzzle[7,8,51].

Most of the cells in a biofilm are highly susceptible to bactericidal agents such as fluoroquinolone antibiotics or metal oxyanions, which can kill both rapidly dividing and slow- or non-growing cells[8,52,53]. This is important, as cells in the biofilm are slow-growing, and many are probably in the stationary phase of growth. The experiment also revealed a small sub-population of cells that remain alive irrespective of the concentration of the antibiotic (persisters). The number of surviving persisters was greater in the non-growing stationary phase. *In vitro*, a stationary culture seems to be more tolerant than a biofilm to antibiotics. However, this situation is probably reversed *in vivo*. Antibiotic treatment will kill most biofilm and planktonic cells, leaving persisters alive. At this point, the similarity with an *in vitro* experiment probably ends. The immune system can mop up and kill remaining planktonic persisters, just as it eliminates non-growing cells of a bacterial population that is treated with a bacteriostatic antibiotic (FIG. 4). However, the biofilm exopolymer matrix protects against immune cells[54–56], and persisters that are contained in the biofilm can survive both the onslaught of antibiotic treatment and the immune system. When the concentration of antibiotic reduces, persisters can repopulate the biofilm, which will shed off new planktonic cells, producing the relapsing biofilm infection[51]. The problem of biofilm resistance to killing by most therapeutics probably defaults to persisters. Interestingly, yeast biofilms also form tolerant persisters (BOX 2).

Is multidrug tolerance transmissible? Virtually all resistance mechanisms can be transferred among bacteria on plasmids or transposons. By contrast, multidrug-tolerance mechanisms that are responsible for persister formation are present in all bacteria, which obviates the need for transmission. However, several observations indicate that drug tolerance is transmissible among populations.

As noted previously, TA modules were first characterized as a plasmid-maintenance mechanism[57]. The toxins can be grouped into two categories — those that rapidly kill the daughter cell that did not receive a plasmid (membrane-acting proteins that cause leaks) and those that cause reversible stasis, such as the translation inhibitors RelE and MazF[26]. Interestingly, the killer toxins improve plasmid maintenance 100–1,000 times, whereas the stasis toxins improve plasmid maintenance by only 4–10 fold. Why retain an ineffective maintenance mechanism when an effective one is available and could easily be acquired by horizontal gene transfer? Perhaps the stasis toxins are maintained by increasing the multidrug tolerance of the host.

There is also an unusual case of transmissible resistance that functions in the same way as tolerance. The plasmid-coded Qnr (Quinolone resistance) proteins confer relatively low-level (but clinically significant)

Box 2 | **Persisters in yeast biofilms**

Yeasts form biofilms, which, in common with bacterial biofilms, are responsible for highly recalcitrant infections[79]. We recently examined the biofilm resistance of *Candida albicans*, following the same approaches that were previously used for bacteria. A dose-dependent experiment with two microbicidal agents, amphotericin B and chlorhexidine, resulted in the complete elimination of cells in exponential and stationary planktonic populations. However, biphasic killing was observed in a mature biofilm, indicating the presence of persisters[80]. In common with bacteria, yeast persisters are not mutants, as on re-inoculation, surviving cells have the same features as the original wild-type population, with a new fraction of persister cells produced. Staining with fluorescein acetate, which specifically binds to dead yeast cells, showed live persisters in a yeast biofilm after treatment with amphotericin B. These rare live cells were either yeast or pseudo-hyphal forms and were morphologically unremarkable. Sorting of this population showed that dim cells were able to form colonies, whereas bright ones were not. This sorting method opens the way for obtaining a gene-expression profile of yeast persisters.

Quite unexpectedly, *C. albicans* persisters were only found in a biofilm culture, and were not present in a non-growing stationary phase population. This specific production of persisters in a biofilm is different from the production of persisters that is observed in bacterial populations, in which a stationary planktonic culture contains more persisters than a biofilm[8]. This might indicate that the biofilm, and not the planktonic population, is the survival mode of yeast life, and that the yeast biofilm is the site of production of specialized survivor cells.

resistance to fluoroquinolones[58,59]. The crystal structure of a Qnr homologue, the chromosomally encoded MfpA of *Mycobacterium tuberculosis*, has been solved. Remarkably, MfpA is a structural DNA mimic that binds to DNA gyrase, the target of fluoroquinolones[60]. The pentapeptide repeat in the Qnr family of proteins produces a helical structure that resembles the double helix of DNA. MfpA binds to, and sequesters, DNA gyrase thereby inhibiting the enzyme and preventing the gyrase/fluoroquinolone complex from nicking the DNA. It would be interesting to determine how much tolerance Qnr proteins add to a cell — how many more cells would survive treatment with a fluoroquinolone if they were expressing one of these proteins?

Persistent infections. Several infections, such as syphilis, lyme disease and tuberculosis (TB), persist for years in the body in an apparently benign form. In most chronic (persistent) infections it seems that the pathogen is at least partially shielded from the immune system — *Treponema pallidum* (syphilis) and *Borrelia burgdorferi* (Lyme disease) migrate into the CNS, whereas *M. tuberculosis* (TB) is hidden in macrophages or granulomas[61]. Are dormant persister cells responsible for the latent, asymptomatic stages of disease? Significant progress has been made towards understanding the metabolic functions that are required for the persistence of *M. tuberculosis*[62], but no persister genes have been isolated for this, or any other, pathogen that causes latent infection. However, it is worth noting that *M. tuberculosis* has at least 60 TA loci [63].

Persisters and antimicrobial therapy

Given the prominent role of tolerance to antibiotics in the aetiology of infectious disease, the need for compounds that could eradicate persisters is obvious. Another important factor to consider is the potential causality between tolerance and antibiotic resistance. A lengthy, lingering infection that is not eradicated owing to tolerance is likely to provide a fertile ground for the emergence of resistant mutants, or for the acquisition of resistance determinants through horizontal gene transfer from other species. A mathematical model predicts that tolerance substantially

increases the incidence of resistance[64]. This observation provides an additional incentive for developing compounds that can sterilize rather than those that merely suppress an infection.

In trying to combat persisters, we might have encountered the ultimate adversary. Indeed, persisters evolved over billions of years to accomplish a single feat — survival. During this time, this cell-type has encountered a huge array of harmful compounds, and the inability of any antibiotic in current use to eliminate persisters provides a sobering view of the magnitude of the challenge to those aiming to develop anti-persister therapies.

An anti-persister drug based on traditional approaches could be produced by combining a conventional antibiotic, such as a fluoroquinolone, and an inhibitor of an essential persister protein. As mentioned previously, a mutation in the essential *E. coli* gene encoding PlsB, which increases the K_m of the PlsB protein, results in a 2–3-log drop in the frequency of persister formation[18]. Proteins such as PlsB that are essential for maintaining the persister state might be attractive targets for anti-persister drug development.

However, unlike conventional antibiotic treatment, anti-persister therapies face an additional hurdle. The food and drug administration (FDA) only require testing against rapidly growing bacteria, and market conditions are excellent for a new conventional broad-spectrum antibiotic. Why then commit resources to the considerably more challenging development of an anti-persister therapy?

A disarmingly simple approach to sterilize an infection was first proposed by Bigger in 1944 (REF. 1). The proposal is to kill bacterial cells with a high dose of an antibiotic, then allow the antibiotic concentration to decrease, which will enable persisters to resuscitate and start to grow. If a second dose of antibiotic is administered shortly after persisters start to grow, a complete sterilization might be achieved. This approach is successful *in vitro*, and a *P. aeruginosa* biofilm can essentially be sterilized with 2 consecutive applications of a fluoroquinolone (K. L., unpublished observations). Perhaps understandably, this approach has not been received with enthusiasm by specialists in clinical microbiology. The

goal of established therapies is to maintain the plasma level of an antibiotic at a maximum concentration, in order to discourage the development of resistance. Most importantly, an optimal pulse-dosing regimen would probably vary from patient to patient. However, it seems that some patients might have inadvertently taken solving the problem of intractable persistent infections into their own hands. Individuals who suffer from persistent infections that require a lengthy therapy are often cured, but why a year-long regimen is better than a month-long one is unclear. An efficacious fluctuating dose of antibiotics administered serendipitously by the patient might be responsible for persister eradication in these cases. The patients might adjust drug dosing simply through being absent-minded, which sooner or later could produce the perfect drug-administration regimen. Curing persistent infections might therefore result from patient non-compliance. Analysing how persistent infections are cured might shed light on the likelihood of developing a rational regimen for the pulse-dosing sterilization of infection.

Persister cells can be killed by antiseptics, but these are obviously toxic and largely unsuitable for systemic applications. The recent development of sterile surface materials[65,66] provides an attractive approach for producing a relatively non-toxic antiseptic by covalently attaching the antimicrobial molecule to the surface. This would prevent leaching of the antiseptic and limit contact with tissue cells. There is an obvious problem with this approach — once attached to the surface, an antimicrobial molecule is immobilized and is unable to reach and kill the pathogen. This problem was solved by linking the antimicrobial compound to a long, flexible polymeric chain that is covalently anchored to the surface of a material. Attaching a long chain of poly-(4-vinyl-*N*-alkylpyridinium bromide) to an amino glass slide (derivatized to contain free-NH$_2$ groups) produced a material that remained largely sterile. Similar immobilized polymers poly [2-(dimethylamino)ethyl methacrylate][67] and *N*-alkyl-polyethylenimine, rapidly depolarized and killed *S. aureus* or *E. coli* cells that came into contact with the surface, leaving no evidence of surviving persisters[68]. Importantly, in order to be effective, the sterile-surface polymers must be long enough to penetrate across the cell envelope[69,70], as shorter versions were ineffective[69].

The attractive properties of antimicrobial polymers are likely to lead to the development of products with sterile surfaces that will prevent the growth of biofilms on catheters and indwelling devices. These materials, however, do not address the need for systemic sterilizing antibiotics.

Is it possible to develop a single-compound anti-persister antibiotic? Known target-specific antibiotics do not sterilize an infection. Antiseptics that can kill persisters do not have specific targets, but damage the cell membrane or macromolecules such as DNA and proteins, and are cytotoxic. Considering this, developing a single-molecule sterilizing antibiotic does not seem feasible. However, let us consider the formation of a perfect antibiotic from first principles (FIG. 5). The

proposed pro-antibiotic compound is benign, but a bacterial enzyme converts it into a reactive antiseptic compound in the cytoplasm. The active molecule cannot be exported owing to increased polarity, and attaches covalently to many bacterial targets, killing the cell. Irreversible binding of the active antibiotic molecule to cellular targets allows the compound to avoid MDR efflux.

Several existing antimicrobials have properties that closely match those of the ideal pro-antibiotic. These are isoniazid, pyrazinamide, ethionamide (anti-*M. tuberculosis* drugs), and metronidazole, a broad-spectrum compound that is active against anaerobic bacteria. Once inside the cell, all four compounds convert into active antiseptic-type molecules that covalently bind to their targets. It seems highly pertinent to this discussion that pro-drug antibiotics comprise the core of the anti-*M. tuberculosis* drug arsenal. *M. tuberculosis* might form the most intransigent persisters, and excellent bactericidal properties are a crucial feature for any anti-TB antibiotic. Preferred targets have been identified for isoniazid and ethionamide[71], which might indicate a relatively limited reactivity of these compounds. The existence of preferred targets indicates that the pro-drug products are not particularly reactive, and that there is considerable scope for the development of better sterilizing antibiotics based on the same principles.

Concluding remarks

The entrance of cells into a dormant, persistent state is largely responsible for the multidrug tolerance of infections. The presence of dormant persisters in biofilms accounts for their tolerance to all known
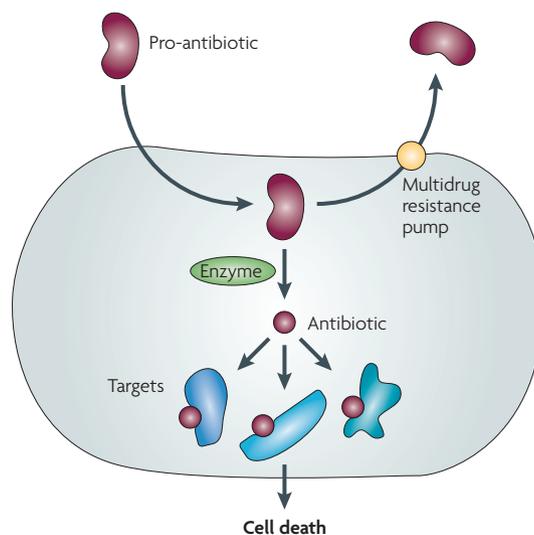
Figure 5 | **The perfect antibiotic.** The pro-antibiotic is benign, but a bacterial enzyme converts it into a reactive antibiotic in the cytoplasm. The active molecule does not leave the cytoplasm (owing to increased polarity), and attaches covalently to many targets, thereby killing the cell. Irreversible binding to the targets prevents the antibiotic from multidrug resistance efflux.

antimicrobials. Persisters are likely to be responsible for latent (chronic) diseases, such as TB, which can be suppressed, but not eradicated, with existing antimicrobials. The need to develop novel therapeutics capable of killing persister cells and eradicating infections is acute. Finding genes responsible for persister formation and maintenance should lead to drugs that disable persisters and might allow conventional antibiotics to eradicate an infection.

The inability of most bacterial species to grow *in vitro* is well-recognized, and it seems likely that uncultivable species might enter into a protective dormant state in unfamiliar environments. Although some important progress has recently been made in the study of persisters and uncultivable bacteria, there is a striking disparity between the importance of the problems outlined in this Review, and the number of professionals working in these fields. This problem is especially prominent in the field of unculturable bacteria, in which the proportion of such species is ≥99%, whereas >99.9% of professionals work

on the 1% of cultivable organisms, and only a minority of scientists are studying the uncultivability issue. Similarly, multidrug tolerance of biofilms is responsible for 65% of all cases of infections in the developed world, but there are probably fewer than ten scientists working on the mechanism underlying this problem. In addition, out of this tiny band of scientists there are probably fewer than ten academics, and none at all working in industry, to tackle the problem of persisters. The significance of the problems outlined above is, however, appreciated in the microbiology community. For example, a recent ASM session outlined the most significant directions for research and bacterial unculturability and biofilms were ranked among the most important topics[72]. Why then is there a reluctance to study problems that are both exciting and important? Taking on the challenges of such recalcitrant problems is fraught with uncertainty. Solving these problems requires patience and risk-taking, both from researchers and from the agencies that support their work.

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