Persister Cells

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Annu. Rev. Microbiol. 2010.64:357-372. Downloaded from www.annualreviews.org
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Annu. Rev. Microbiol. 2010. 64:357–72

First published online as a Review in Advance on June 9, 2010

The *Annual Review of Microbiology* is online at micro.annualreviews.org

This article's doi:
10.1146/annurev.micro.112408.134306

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0066-4227/10/1013-0357$20.00

Key Words

antibiotic, antimicrobial tolerance, biofilm, resistance, stress response, stochasticity

Abstract

Persisters are dormant variants of regular cells that form stochastically in microbial populations and are highly tolerant to antibiotics. High persister (*hip*) mutants of *Pseudomonas aeruginosa* are selected in patients with cystic fibrosis. Similarly, *hip* mutants of *Candida albicans* are selected in patients with an oral thrush biofilm. These observations suggest that persisters may be the main culprit responsible for the recalcitrance of chronic infectious disease to antimicrobial therapy. Screening knockout libraries has not produced mutants lacking persisters, indicating that dormancy mechanisms are redundant. Toxin/antitoxin (TA) modules are involved in persister formation in *Escherichia coli*. The SOS response leads to overexpression of the TisB toxin and persister formation. TisB is a membrane-acting peptide that apparently sends cells into dormancy by decreasing the proton motive force and ATP levels. Stress responses may act as general activators of persister formation. Proteins required for maintaining persisters may represent realistic targets for discovery of drugs capable of effectively treating chronic infections.

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A BRIEF HISTORY

Persisters play a major role in the recalcitrance of chronic infections to antibiotics. They were discovered 66 years ago, yet we are only now beginning to understand the mechanism of their formation. Why the disparity between the significance of this very old problem and the modest pace of progress in the field? This is the question we will ponder in reviewing the history of the problem.

Persisters: cells that entered a dormant, multidrug-tolerant state

Antimicrobial tolerance: a property of dormant cells that survive killing by bactericidal antibiotics in the absence of drug resistance mechanisms

Antimicrobial resistance: an ability to prevent the interaction of an antibiotic with a target by a variety of resistance mechanisms

In 1944, Joseph Bigger, a medical doctor from the University of Dublin, was working in a military hospital in York, experimenting with the recently introduced penicillin. Addition of penicillin to a culture of *Staphylococcus* resulted in lysis (11). Bigger nonetheless plated this transparent liquid and recorded surviving colonies. Upon reinoculation, these colonies grew into a culture that again lysed in the presence of penicillin but formed a small new subpopulation of what Bigger dubbed "persisters" to differentiate them from resistant mutants. Many years later, we repeated this experiment with *Escherichia coli* and obtained the same result (**Figure 1**) (46). Bigger concluded, with regret, that penicillin did not have the ability to sterilize an infection. As we now know, this deficiency is shared by all antibiotics due to persister cell tolerance (**Figure 2**).

Shortly after the introduction of penicillin, antimicrobial resistance to the drug was reported and was eventually traced to the ability of pathogens to produce β-lactamases that destroy the antibiotic. The spread of drug resistance threatened to undermine the effectiveness of the newly discovered antibiotics, and the study of resistance became of paramount importance. The curious case of persister cell tolerance was promptly forgotten.

The golden era of antibiotic discovery, from the 1940s through the 1960s (89), produced many effective compounds, outpacing the spread of resistance. It also became apparent that resistance, although important (55), does not render antibiotics useless. Introduced in the 1940s through the 1950s, penicillin, streptomycin, and tetracycline, for example, are still in use. The temporary success in the standoff with pathogens prompted a declaration of victory from the Surgeon General in 1967

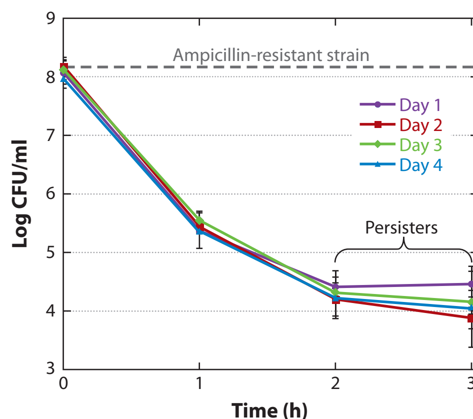

Figure 1

A test for persister heritability. An exponentially growing culture of *Escherichia coli* was treated with a high dose of ampicillin at time 0. After lysis, surviving persisters were reinoculated in fresh medium, cultured, and ampicillin was applied again. The dashed line indicates how an ampicillin-resistant strain would have behaved. Based on Reference 46.

("time to close the book on infectious diseases" and "declare the war against pestilence won, and shift national resources to such chronic problems as cancer and heart disease"), and the subject of microbes and antibiotics was deprioritized. After a 40-year gap, Harris Moyed picked up the problem of persisters (12, 13, 64, 65, 74); he was apparently motivated by an interest in nonheritable variation, not by the need to understand an important human health issue. Moyed selected for mutants with increased levels of persisters by repeatedly exposing growing cells of *E. coli* to ampicillin. Any mutants that could grow in the presence of antibiotic (i.e., had a higher MIC) were discarded. This led to the identification of a *hipA*7 gain-of-function allele (65, 49). A *hipA* deletion, however, had no phenotype, questioning the role of HipA in the formation of wild-type persisters. This line of inquiry seemed to be reaching a dead end. The pioneering work of Moyed, and persisters, were forgotten, this time for only a decade.

By the 1990s, it became clear that our victory over pathogens was an illusion, with resistance spreading faster than the discovery of new antibiotics. Apart from acute infections, chronic infections were on the rise owing to several factors: the widespread use of indwelling devices and the increase in immunocompromised patients due to cancer chemotherapy and HIV. Many chronic infections were associated with the ability of the pathogen to form a biofilm (19). Biofilms show a surprising ability to resist killing by antibiotics, without having any obvious drug resistance mechanisms. Our study of dose-dependent killing of a *Pseudomonas aeruginosa* biofilm showed the presence of a small subpopulation of cells completely tolerant to antibiotics (14, 82). The rediscovery of persisters in biofilms pointed to the likely culprit of antibiotic recalcitrance and renewed an interest in their study. The advances in studying persisters that were obtained in the last decade have led to the understanding that persisters are dormant cells (5, 77), and methods to isolate and analyze them have been established. The first mechanism of persister formation has

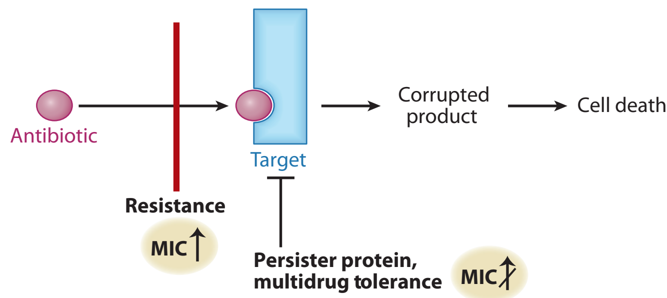

Figure 2

Resistance versus tolerance. Bactericidal antibiotics kill cells by forcing the active target to produce corrupted products. Streptomycin interrupts translation, producing toxic misfolded peptides (21). Inhibition of peptidoglycan synthesis by β-lactams causes induction of autolysins and cell death by a process that is still poorly understood (9); fluoroquinolones bind to the DNA gyrase and convert it to DNA endonuclease (41). A recent study reported that bactericidal antibiotics also lead to the production of reactive oxygen species, contributing to cell death (48). Persister proteins act by blocking the target, so no corrupted product can be produced. By contrast, all resistance mechanisms prevent the antibiotic from binding to the target.

been identified, but there is little doubt that the dormancy programs are highly redundant.

The most important question regarding persisters is the same as for any subject—What is its significance? Persisters seem to be the main reason for the recalcitrance of chronic infections to antimicrobials. The main problem then is establishing causality.

FROM PLAUSIBILITY TO CAUSALITY: LINKING PERSISTERS TO DISEASE

Infectious disease is often untreatable, even when caused by a pathogen that is not resistant to antibiotics. This is the essential paradox of chronic infections. In most cases, chronic infections are accompanied by the formation of biofilms, which seems to indicate the source of the problem (19, 23). Biofilms have been linked to dental disease, endocarditis, cystitis, urinary tract infections, deep-seated infections, indwelling device and catheter infections, and the incurable disease of cystic fibrosis. In the case of indwelling devices such as prostheses and heart valves, reoperation is the method of choice for treating the infection. Biofilms do not generally restrict penetration of antibiotics

hip: high persister mutants

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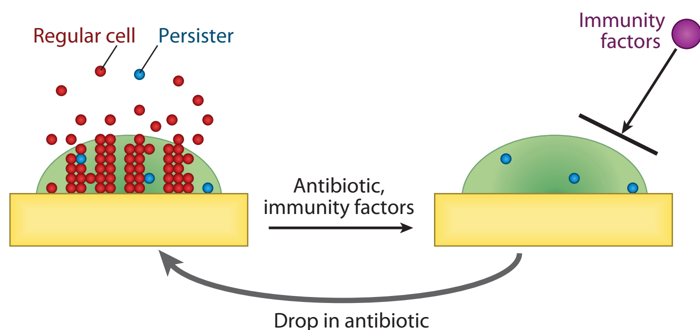

Figure 3

A model of a relapsing biofilm infections. Regular cells and persister cells form in the biofilm and are shed off into surrounding tissue and the bloodstream. Antibiotics kills regular cells, and the immune system eliminates escaping persister cells. The matrix protects persister cells from the immune system, and when the concentration of the antibiotic drops, they repopulate the biofilm, causing a relapse.

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(90), but they do form a barrier for the larger components of the immune system (43, 54, 88). The presence of biofilm-specific resistance mechanisms was suggested to account for the recalcitrance of infectious diseases (84). However, the bulk of cells in the biofilm are actually highly susceptible to killing by antibiotics; only a small fraction of persisters remains alive (82). On the basis of these findings, we proposed a simple model of a relapsing chronic infection: Antibiotics kill the majority of cells, and the immune system eliminates both regular cells and persisters from the bloodstream (57) (**Figure 3**). The only remaining live cells then are persisters in the biofilm. Once the level of antibiotic drops, persisters repopulate the biofilm, and the infection relapses. While this is a plausible model, it is not the only one. A simpler possibility is that antibiotics fail to effectively reach at least some cells in vivo, resulting in a relapsing infection.

CF: cystic fibrosis

Establishing potential causality between persisters and therapy failure is not trivial, because these cells form a small subpopulation with a temporary phenotype, which precludes introducing them into an animal model of infection. We reasoned that causality can be tested based on what we know about selection for high persister (*hip*) mutants in vitro. Periodic application of high doses of bactericidal antibiotics leads to the selection of strains that produce increased levels of persisters (64, 92). This is precisely what happens in the course of treating chronic infections—the patient is periodically exposed to high doses of antibiotics, which may select for *hip* mutants. But *hip* mutants would only gain advantage if the drugs effectively reach and kill the regular cells of the pathogen.

Patients with cystic fibrosis (CF) are treated for decades for an incurable *P. aeruginosa* infection to which they eventually succumb (32). The periodic application of high doses of antibiotics provides some relief by decreasing the pathogen burden, but it does not clear the infection. If *hip* strains of pathogens were selected in vivo, they would most likely be present in a CF patient. We took advantage of a set of longitudinal *P. aeruginosa* isolates from a single patient collected over the course of many years (80). Testing persister levels by monitoring survival after challenge with a high dose of ofloxacin showed a dramatic, 100-fold increase in surviving cells in the last four isolates (66). Testing paired strains from additional patients showed that in most cases there was a considerable increase in persister levels in the late isolate from a patient. Most of the *hip* isolates had no increase in MIC compared with their clonal parent strain to ofloxacin, carbenicillin, and tobramycin, suggesting that classical acquired resistance plays little to no role in the recalcitrance of CF infection. These experiments directly link persisters to the clinical manifestation of the disease and suggest that persisters are responsible for the therapy failure of chronic CF infection. But why have the *hip* mutants with their striking survival phenotype evaded detection for such a long time?

The main focus of research on antimicrobials has been on drug resistance, and the basic starting experiment is to test a clinical isolate for its ability to grow in the presence of elevated levels of different antibiotics, and record any increases in the MIC (see sidebar, Resistance Versus Persister Tolerance). This is also the standard test employed by clinical microbiology laboratories. *hip* mutants are of course

missed by this test, which explains why they had remained undetected, in spite of a major effort aimed at understanding pathogen survival to antimicrobial chemotherapy. Given that *hip* mutants are likely the main culprit responsible for morbidity and mortality of CF infection, it makes sense to test for their presence. Testing for persister levels is not that much more difficult than performing an MIC test.

Is selection for *hip* mutants a general feature of chronic infections? We recently examined patients with chronic oral thrush caused by *Candida albicans* (53). These were cancer patients undergoing chemotherapy, and suppression of the immune system caused the fungal infection. In patients for whom the disease did not resolve, the *C. albicans* isolates were almost invariably *hip* mutants, compared with patients for whom the disease cleared within 3 weeks of treatment with chlorhexidine. The eukaryotic *C. albicans* forms persisters (1, 37, 52) through mechanisms that are probably analogous, rather than homologous, to those of their bacterial counterparts. Given the similar lifestyles of the unrelated *P. aeruginosa* and *C. albicans*, we may expect that the survival advantage of a *hip* mutation is universal. Just as multidrug resistance has become the prevalent danger in acute infections, multidrug tolerance of persisters and *hip* mutants may be the main, but largely overlooked, culprit of chronic infectious disease.

Biofilms apparently serve as a protective habitat for persisters (35, 36, 38, 52, 82), allowing them to evade the immune response. However, a more general paradigm is that persisters are critical for pathogens to survive antimicrobial chemotherapy whenever the immune response is limited. Such cases would include disseminating infections in immunocompromised patients undergoing cancer chemotherapy or infected with HIV. Persisters are also likely to play an important role in immunocompetent individuals in cases where the pathogen is located at sites poorly accessible by components of the immune system. These include the central nervous system, where pathogens cause debilitating meningitis and brain abscesses (40), and the gastrointestinal tract, where the hard-to-eradicate *Helicobacter pylori* causes gastroduodenal ulcers and gastric carcinoma (70). Tuberculosis is perhaps the most prominent case of a chronic infection by a pathogen evading the immune system. The acute infection may resolve spontaneously or as a result of antimicrobial therapy, but the pathogen often remains in a latent form (7). It is estimated that 1 in every 3 people carry latent *Mycobacterium tuberculosis*, and 10% of carriers develop an acute infection at some stage in their lives. Virtually nothing is known about this latent form that serves as the main reservoir of tuberculosis. One simple possibility is that persisters are equivalent to the latent form of the pathogen. Several groups, including ours, are currently working on persister isolation from *M. tuberculosis*.

RESISTANCE VERSUS PERSISTER TOLERANCE

Persisters represent a small subpopulation of cells that spontaneously enter a dormant, nondividing state. When a population is treated with a bactericidal antibiotic, regular cells die, whereas persisters survive. In order to kill, antibiotics require active targets, which explains persister tolerance. Taking samples and plating them for colony counts over time from a culture treated with antibiotic produces a biphasic pattern, with a distinct plateau of surviving persisters. By contrast, resistance mechanisms prevent antibiotics from binding to their targets. Resistance is measured by observing the ability of cells to grow in the presence of antibiotic. In order to measure resistance, antibiotic is serially diluted in twofold steps in a microtiter plate containing cells and growth medium. After a period of incubation, the lowest concentration of the antibiotic in a well with no growth is recorded and referred to as an MIC.

MECHANISM OF PERSISTER FORMATION

Screening Knockout Libraries in Search of Persister Genes

The most straightforward approach to finding an underlying mechanism of a complex

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function is by screening a library of transposon (Tn) insertion mutants. This produces a set of candidate genes, and subsequent analysis leads to a pathway and a mechanism. This is indeed how the basic mechanisms of sporulation, flagellation, chemotaxis, virulence, and many other functions have been established. However, screening a Tn insertion library of *E. coli* for an ability to tolerate high doses of antibiotics produced no mutants completely lacking persisters (42, 81). This led those in the field to rethink the standard one phenotype/one mechanism model. One interesting hypothesis is that there is no mechanism of persister formation (86). Rather, accidental formation of various misfolded proteins causes stasis, producing persisters. Ectopic expression of the chaperone DnaJ from *E. coli* or PmrC, a *S. enterica* enzyme, both toxic when overproduced in *E. coli*, inhibited cell growth and resulted in multidrug tolerance, supporting this hypothesis. However, this explanation is inconsistent with what we know about the dynamics of persister formation. Very few persisters are present in an early exponential culture and then rise sharply at around mid-exponential state (46). Maintaining a population in early exponential state by serial reinoculation resulted in complete disappearance of persisters (46). This means that no persisters are formed during early exponential growth; those that are present initially are leftovers from stationary state and are diluted out by repeated reinoculation. This also means that persisters are formed likely by dedicated mechanisms than through random mistakes in protein misfolding, which would be expected to occur throughout the growth of the culture.

With the development of the complete, ordered *E. coli* gene knockout library by the Mori group [(4) the Keio collection], it seemed reasonable to revisit the screening approach. Indeed, there always remains a possibility that transposons missed a critical gene or that the library was not large enough. The use of the Keio collection largely resolves this uncertainty.

This advanced screen (34), similar to previous efforts, did not produce a single mutant lacking persisters, suggesting a high degree of redundancy. The screen did identify a number of interesting genes, with knockouts showing about a 10-fold decrease in persister formation. The majority of hits were in global regulators, DksA, DnaKJ, HupAB, and IhfAB. This is an independent indication of redundancy—a global regulator can affect expression of several persister genes simultaneously, resulting in a phenotype (**Figure 4**). The screen also produced two interesting candidate genes that may be more directly involved in persister formation: YgfA, which can inhibit nucleotide synthesis, and YigB, which may block metabolism by depleting the pool of flavin mononucleotide.

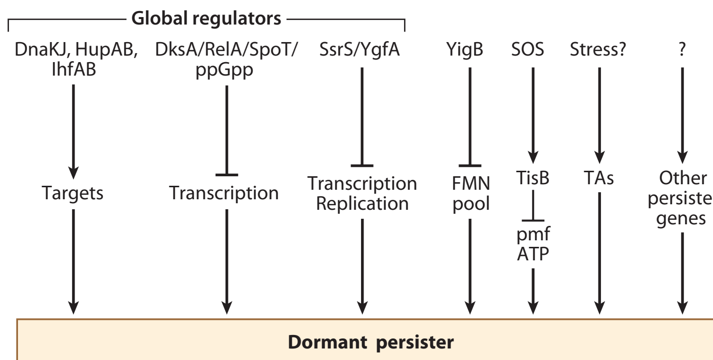

Figure 4

Redundant pathways of persister formation. Candidate persister genes and their targets, when known, are indicated. Abbreviations: FMN, flavin mononucleotide; pmf, proton motive force; TA, toxin/antitoxin modules.

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A similar screen of a *P. aeruginosa* mutant library was recently reported (22). As in *E. coli*, no persisterless mutant was identified, pointing to the similar redundancy theme.

The main conclusion from the screens is that persister formation does not follow the main design theme of complex cellular functions, i.e., a single linear regulatory pathway controlling an execution mechanism. By contrast, persisters are apparently formed through a number of independent parallel mechanisms (**Figure 4**). There is a considerable adaptive advantage to this redundant design: No single compound will disable persister formation. It is interesting to consider in this regard the targets of antibiotics. The most common antibiotic, produced by 10% of all *Streptomyces* species, is streptothricin, an aminoglycoside that binds to the 16S ribosomal RNA. 16S RNA is the most conserved gene in living organisms, and we take advantage of this conservation to build evolutionary trees based on it. Bacteria take advantage of this conservation to kill as many competitors as possible. The next most abundant antibiotic is streptomycin, another aminoglycoside targeting 16S RNA (1% incidence), followed by tetracycline, targeting the ribosome at 0.01–0.1 incidence, and so on (6). The redundancy of persister mechanisms and the poor conservation will then preclude both natural and synthetic production of compounds targeting them.

Screens for persister genes were useful in finding some possible candidate genes and in pointing to redundancy of function. It seemed that a method better suited to uncover redundant elements would be transcriptome analysis, for which persisters had to be isolated.

Persister Isolation and Transcriptome Analysis

Persisters form a small and temporary population, making isolation challenging. The simplest approach is to lyse a population of growing cells with a β-lactam antibiotic and collect surviving persisters (47). This isolates enough *E. coli* cells to perform a transcriptome analysis. Although persisters are not killed by the antibiotic, this is not an indifferent factor, and the surrounding environment changes drastically as the bulk of the population lyses. While the resulting transcriptome is not pristine, it is nonetheless useful. A more advanced method aimed at isolating native persisters was developed, based on a guess that these are dormant cells with diminished protein synthesis (77). If the strain expressed degradable GFP, then cells that stochastically enter into dormancy will become dim (**Figure 5**). In a population of *E. coli* expressing degradable GFP under the control of a ribosomal promoter that is only active in dividing cells, a small number of cells indeed appeared to be dim (**Figure 5**). The difference in fluorescence allowed the two subpopulations to be sorted. The dim cells were tolerant to ofloxacin, confirming that they are persisters. Although this method should be generally applicable to sorting persisters of any species, it has its limitations. Sorting dilutes the population into buffer, changing both the cell concentration and the medium. Dilution leads to resuscitation, resulting in a decrease in persister levels. These problems notwithstanding, a transcriptome of sorted persisters was obtained. Transcriptomes obtained by both methods pointed to downregulation of biosynthesis genes and indicated increased expression of several toxin/antitoxin (TA) modules (RelBE, MazEF, DinJYafQ, YgiU).

TA: toxin/antitoxin modules

TA Modules and Dormancy

TA modules are found on plasmids, where they constitute a maintenance mechanism (31, 39). 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 due 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. In *E. coli*, MazF

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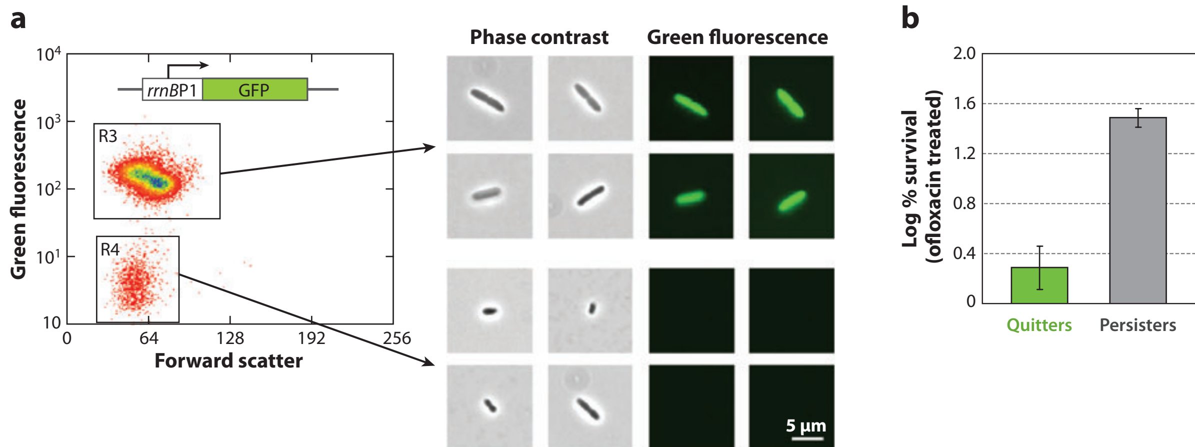

Figure 5

Sorting of persister cells. (*a*) An *Escherichia coli* strain expressing a degradable GFP under the control of a ribosomal promoter was cultured to exponential state, and the cells were sorted based on the green signal. The larger green population is made of regular cells, and the small subpopulation is made of dim cells that apparently have diminished translation. (*b*) Exposure of these two subpopulations to antibiotics shows that the dim fraction is made of tolerant persisters.

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and an unrelated toxin, RelE, induce stasis by cleaving mRNA, which inhibits translation, a condition that can be reversed by expression of corresponding antitoxins (16, 68). This property of toxins makes them excellent candidates for persister genes.

Ectopic expression of RelE (47) or MazF (86) strongly increased tolerance to antibiotics. The first gene linked to persisters, *hipA* (64), is also a toxin, and its ectopic expression causes multidrug tolerance as well (18, 27, 50, 86). A bioinformatics analysis indicates that HipA is a member of the Tor family of kinases, which have been extensively studied in eukaryotes (75) but have not been previously identified in bacteria. HipA is indeed a kinase; it autophosphorylates on Ser150, and site-directed mutagenesis replacing it, or other conserved amino acids in the catalytic and Mg^{2+}-binding sites, abolishes its ability to stop cell growth and confer drug tolerance (18). The crystal structure of HipA in complex with its antitoxin HipB was recently resolved, and a pull-down experiment showed that the substrate of HipA is elongation factor EF-Tu (76). Phosphorylated EF-Tu is inactive, which leads to a block in translation and dormancy.

Deletion of potential candidates of persister genes noted above does not produce a discernible phenotype affecting persister production, possibly owing to the high degree of redundancy of these elements. In *E. coli*, there are at least 15 TA modules (2, 67, 69), and more than 80 in *M. tuberculosis* (72). Several TA modules are upregulated in *M. tuberculosis* in response to stress factors (72), and the effects of overexpression versus deletion of the three RelE homologs of this pathogen were examined (78). The RelE homologs of *M. tuberculosis* affected survival to particular drugs but did not affect multidrug tolerance. It is also unclear whether the effects of RelE homologs were directed against the bulk of the population or specifically against persisters. More studies are required to investigate the effect of the super-redundant TA modules on *M. tuberculosis* persisters.

High redundancy of TA genes would explain the lack of a multidrug-tolerant phenotype in knockout mutants, and therefore it seemed useful to search for conditions in which a particular toxin would be highly expressed in a wild-type strain, and then examine a possible link to persister formation.

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The TA modules *symER*, *hokE*, *yafN/yafO*, and *tisAB/istr1* contain the Lex box and are induced by the SOS response (20, 28, 45, 62, 63, 69, 79, 87). Fluoroquinolones induce the SOS response (71), and we tested the ability of ciprofloxacin to induce persister formation (24).

Examination of deletion strains showed that the level of persisters dropped dramatically, from 10- to 100-fold, in a Δ*tisAB* mutant (25). This suggests that TisB was responsible for the formation of the majority of persisters under conditions of SOS induction. The level of persisters was unaffected in strains deleted in the other Lex-box-containing TA modules. Persister levels observed in time-dependent killing experiments with ampicillin or streptomycin that do not cause DNA damage were unchanged in the Δ*tisAB* strain. TisB only had a phenotype in the presence of a functional RecA protein, confirming the dependency on the SOS pathway.

Ectopic overexpression of *tisB* sharply increased the level of persisters. A decrease in persisters in a deletion strain and increase upon overexpression give reasonable confidence in the functionality of a persister gene. The dependency of TisB-induced persisters on a particular regulatory pathway, the SOS response, further strengthens the case for TisB as a specialized persister protein. Incidentally, a *tisB* mutant is not present in the otherwise fairly complete Keio knockout library, and the small open reading frame might have been easily missed by Tn mutagenesis as well, evading detection by the generalized screens for persister genes.

The role of TisB in persister formation is unexpected based on what we know about this type of protein. TisB is a small, 29-amino-acid hydrophobic peptide that binds to the membrane and disrupts the proton motive force (pmf), which leads to a drop in ATP levels (85). Bacteria, plants, and animals all produce antimicrobial membrane-acting peptides (29, 73, 92). Toxins of many TA loci found on plasmids belong to this type as well. If a daughter cell does not inherit a plasmid, the concentration of a labile antitoxin decreases, and the toxin such as the membrane-acting *hok* kills the cell (30).

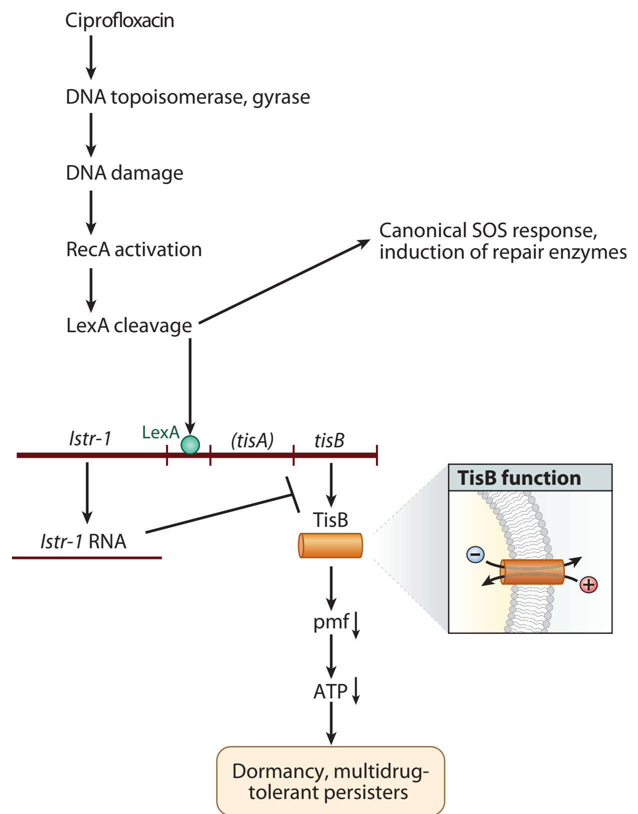

Figure 6

A model of TisB-dependent persister formation in *Escherichia coli*. A fluoroquinolone antibiotic causes DNA damage by converting the DNA gyrase and topoisomerase to endonucleases. This activates the RecA protein, which in turns activates the LexA repressor, causing it to cleave. The canonical SOS response is induced, and repair enzymes that contain *lex* boxes in their promoter regions are transcribed. The Lex repressor also controls the expression of the TisB toxin, a small cationic membrane-acting agent. Decrease in the proton motive force (pmf) and ATP shuts down target functions, including DNA topoisomerase and gyrase, and a dormant persister is formed.

High-level artificial overexpression of TisB also causes cell death (85). It is remarkable from this perspective that the membrane-acting TisB under conditions of natural (mild) expression has the exact opposite effect of protecting the cell from antibiotics (**Figure 6**).

Induction of persisters by the SOS-induced TisB toxin links together two seemingly opposite strategies of survival: active repair and systems shutdown in a dormant state. It seems that in the presence of DNA-damaging factors, the optimal strategy is to both induce

pmf: proton motive force

ANTIBIOTICS CAN INDUCE FORMATION OF MULTIDRUG-TOLERANT PERSISTERS

Fluoroquinolones such as ciprofloxacin are widely used broad-spectrum antibiotics, and their ability to induce multidrug-tolerant cells is unexpected and a cause for considerable concern. Induction of persister formation by fluoroquinolones may contribute to the ineffectiveness of antibiotics in eradicating infections. Indeed, pre-exposure with a low dose of ciprofloxacin drastically increased tolerance to subsequent exposure with a high dose, and TisB persisters are multidrug tolerant.

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repair and increase the number of dormant cells, which will survive when repair fails. Indeed, a progressive increase in the exposure to fluoroquinolones kills regular cells but has little effect on the survival of persisters. This means that the dormant persisters rather than regular cells with induced repair will ultimately survive the DNA-damaging antibiotic.

The discovery of TisB's role in tolerance opens an intriguing possibility of a wider link between other stress responses and persister formation. Pathogens are exposed to many stress factors in the host environment apart from DNA-damaging agents, such as oxidants, high temperature, low pH, and membrane-acting agents. It is possible that all stress responses induce the formation of surviving persisters.

STOCHASTIC AND DETERMINISTIC COMPONENTS OF PERSISTER FORMATION

Persisters make up a small subpopulation and in mid-log-phase *E. coli* or *P. aeruginosa* produce as little as 10^{-5} surviving tolerant cells. Given that all the cells in a population are genetically identical kin, it seems that persisters are produced by a stochastic process (47, 56, 59). Indeed, what would be the alternative? Fluctuations in the levels of a small number of dedicated proteins are probably responsible for persister formation. At the same time, there is a dramatic increase in persister levels from mid-exponential to stationary phases of growth in all species tested so far (46, 82). This means that there is also a deterministic component controlling persister formation. The sharp increase in persister levels while the culture is still growing exponentially resembles the dynamics of quorum sensing (8). However, addition of spent medium to an early exponential culture of *E. coli* failed to increase the level of persisters (K. Lewis, unpublished data). The nature of the factor(s) responsible for persister rise remains to be determined. The role of the SOS response in persister formation provides a clear example of a deterministic component. SOS induces expression of the TisB protein, which in turn causes the formation of dormant persisters. Ectopic expression of TisB inhibits the growth of the entire population, showing that the deterministic component can convert all cells to persister cells. This, however, does not happen under conditions of the SOS response—only a small number of cells become persisters. Apparently, expression of TisB during the SOS response fluctuates around a certain mean level, and cells that stochastically achieve high expression become persisters.

Stochasticity: random fluctuations in gene expression leading to formation of cells that differ from the bulk of the population

Stochastic phenomena have been described for a large number of functions in both bacteria (3, 26) and eukaryotes (44). In *B. subtilis*, stochastic processes are responsible for determining the part of the population that enters sporulation (17), cannibalism (33), competence (61), and biofilm formation (15). The most visible case of bacterial stochasticity is chemotaxis, where stochastic changes in the direction of rotation of a flagellar bundle determine a trial-and-error random walk (10). The probability of a run is increased if there is a temporal increase in the concentration of an attractant. Apart from this stochastic noise within a given cell, individual cells vary dramatically in their run/tumble probability ratio (83). Two particular proteins, CheZ and CheY, of the many elements involved in the chemotaxis signal transduction appear to be responsible for this noise generation (51).

Bacteria apparently use stochastic processes in decision making when presented with a

problem lacking a unique solution. The bacterial cell is too small to have a significant measurable difference between concentrations of a chemoeffector along its length, and trial-and-error runs solve the problem—a temporal increase in the concentration of an attractant increases the duration of the run. And when a population needs to produce a small number of specialized survivor cells, fluctuations in the level of persister proteins induce a dormant state.

There is another level of noise in persister formation that we do not currently understand. It is the distinct variation in persister levels among populations grown under seemingly identical conditions in parallel test tubes or wells of a microtiter plate (91; K. Lewis, unpublished data). It seems premature to speculate about the source of this surprising variation, but its adaptive value may be significant. Just as variability of cells within a population increases the chances of kin survival, variability of persister levels among populations will be similarly adaptive.

UNANSWERED QUESTIONS AND PERSPECTIVES

Despite their discovery more than 60 years ago, the study of persister cells is still in its infancy due to a combination of two factors: uncertain significance and redundancy of mechanisms. For a long time, persisters have been a curiosity for the few microbiologists who knew about them. The principal role of persisters in biofilm antibiotic tolerance in vitro (35, 36, 82) and the recent findings linking persisters to chronic infections in cystic fibrosis (66) and oral thrush (52) suggest that persisters are responsible for the recalcitrance of chronic infections. This largely addresses the significance question, and future studies will detail the role of persisters in all chronic infections. The scope of this considerable task is comparable to what has been done for determining the role of drug resistance in acute infections. We anticipate that testing for persisters and *hip* mutants will become routine in the study and treatment of chronic infections. The other big remaining questions are the mechanism of persister formation and the linked problem of persister eradication. The finding of SOS-TisB-dependent persister formation provides the first precedent for a persister mechanism. This is an exciting time for the field. It is at a stage where the foundation has been built, but major discoveries are still waiting in the wings. These characters may include the following:

- Persister genes. The biggest obstacle in understanding the mechanism of persister formation is the lack of an efficient method to identify persister genes. Standard molecular biology approaches work poorly for finding genes with redundant functions. One promising new approach is whole-genome sequencing of *hip* mutants. But a *hip* mutation may very well create persisters in a way that has nothing in common with the natural mechanism. Overexpression of misfolded proteins produces stasis emulating persisters (86). This means that some if not most *hip* mutations are likely to be artifacts. However, if *hip* mutants are obtained from clinical isolates, then understanding their mechanism of formation is important and the line between fact and artifact becomes blurred.
- *tisB*. Another promising approach stems from the study of the first properly validated persister gene, *tisB*. Under conditions of SOS response, most persisters are formed in a TisB-dependent manner. This reduces the redundancy to one major player. It is possible that other persister genes are induced under particular conditions, such as stress responses, which should enable them to be identified.
- The role of persisters in survival in the external environment. So far, the majority of studies have been restricted to the understanding of drug tolerance. Persisters are tolerant to heavy metals (35, 36), but other factors remain to be examined. The sharp increase in persister levels in stationary state is akin to

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sporulation, which also prepares the population to survive harsh environmental conditions. It is likely that persisters, similar to spores, play a major role as a traveling and surviving form of an organism.

- Resuscitation. We know that persisters resuscitate, but know nothing about its mechanism.
- Persister eradication. This is a tough problem. Very few existing antibiotics are active against nongrowing stationary cells (primarily fluoroquinolones), and all are ineffective against dormant persisters. Persisters are specialized survivor cells and with multiple mechanisms of formation do not provide a good target that would disable their formation. One possible approach is to look for the "Achilles heel" of persisters—not a persister mechanism, but rather a protein essential for maintaining the dormant cells. One example is the need to repair and maintain the membrane, where the fragile lipid bilayer can be rapidly disrupted by oxidation of fatty acids. Indeed, a decrease in the function of PlsB catalyzing the first step in phospholipid biosynthesis decreases persister formation (81). Another possible target is the global regulator PhoU. A knockout strain is sick and was reported to be eradicated by antibiotics with no surviving persisters (60). Combining a regular antibiotic with a compound disabling a persister maintenance function could then provide a sterilizing therapeutic. Another possibility is to develop prodrugs, compounds that enter into the cell and are converted to a generally reactive compound by a bacteria-specific enzyme. The active species will then attack unrelated targets, including DNA and the membrane, and kill dormant cells (58).

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SUMMARY POINTS

1. Microbial populations harbor persisters, which are dormant variants of regular cells that exhibit multidrug tolerance.
2. High persister mutants of *P. aeruginosa* are selected for in the course of infection in patients with cystic fibrosis. High persister mutants of *C. albicans* are selected for in patients with oral thrush. Persisters are likely responsible for the recalcitrance of chronic infectious diseases to antimicrobial chemotherapy.
3. Screens of knockout libraries do not identify mutants completely lacking the ability to form persisters, indicating that the mechanisms of dormancy are redundant.
4. Persisters can be isolated by lysis with antibiotics or by sorting, based on the lack of expression of degradable GFP.
5. TA modules are involved in persister formation.
6. The SOS stress response activates persister formation. Fluoroquinolone antibiotics induce the SOS response, which turns on expression of the TisB toxin, causing dormancy.
7. Persisters are formed through a combination of stochastic and deterministic events.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

Work from the author's laboratory described in this review has been supported by grants T-R01AO085585 and R01 GM061162-05A1 from the NIH. Dr. Iris Keren is acknowledged for helpful discussion, and the author is grateful to Dr. Susan Gottesman for excellent editorial comments.

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