# Persister Cells: Molecular Mechanisms Related to Antibiotic Tolerance

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## Contents

1 Clinical Significance of Persister Cells .................................................. 122
2 Molecular Mechanisms of Dormancy .................................................. 126
References .................................................. 130

**Abstract** It is a given that new antibiotics are needed to combat drug-resistant pathogens. However, this is only a part of the need—we actually never had antibiotics capable of eradicating an infection. All pathogens produce a small subpopulation of dormant persister cells that are highly tolerant to killing by antibiotics. Once an antibiotic concentration drops, surviving persisters re-establish the population, causing a relapsing chronic infection. Persisters are especially significant when the pathogen is shielded from the immune system by biofilms, or in sites where the immune components are limited—in the nervous system, the stomach, or inside macrophages.

Antibiotic treatment during a prolonged chronic infection of *P. aeruginosa* in the lungs of patients with cystic fibrosis selects for high-persister (*hip*) mutants. Similarly, treatment of oral thrush infection selects for *hip* mutants of *C. albicans*. These observations suggest a direct causality between persisters and recalcitrance of the disease. It appears that tolerance of persisters plays a leading role in chronic infections, while resistance is the leading cause of recalcitrance to therapy in acute infections. Studies of persister formation in E. coli show that mechanisms of dormancy are highly redundant. Isolation of persisters produced a transcriptome which suggests a dormant phenotype characterized by downregulation of energy-producing and biosynthetic functions. Toxin–antitoxin modules represent a major mechanism of

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A.R.M. Coates (ed.), *Antibiotic Resistance*, Handbook of Experimental Pharmacology 211, DOI 10.1007/978-3-642-28951-4_8,
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persister formation. The RelE toxin causes dormancy by cleaving mRNA; the HipA toxin inhibits translation by phosphorylating elongation factor Ef-Tu, and the TisB toxin forms a membrane pore, leading to a decrease in pmf and ATP.

**Keywords** Biofilm • Drug tolerance • High-persister mutants • Persister • Toxin/antitoxins

## 1 Clinical Significance of Persister Cells

Persisters represent a small subpopulation of cells that spontaneously go into a dormant, non-dividing state. When a population is treated with a bactericidal antibiotic, regular cells die, while persisters survive (Fig. 1). In order to kill, antibiotics require active targets, which explains tolerance of persisters. 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 (Fig. 2).

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 point to the source of the problem (Costerton et al. 1999; Del Pozo and Patel 2007). Biofilms have been linked to dental disease, endocarditis, cystitis, UTI, 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 (Walters et al. 2003), but do form a barrier for the larger components of the immune system (Jesaitis et al. 2003; Leid et al. 2002; Vuong et al. 2004). The presence of biofilm-specific resistance mechanisms was suggested to account for the recalcitrance of infectious diseases (Stewart and Costerton 2001). However, the bulk of cells in the biofilm are actually highly susceptible to killing by antibiotics; only a small fraction of persisters remains alive (Spoering and Lewis 2001). Based on 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 (Lewis 2001) (Fig. 3). The only remaining live cells are then 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.

Establishing potential causality between persisters and therapy failure is not trivial, since 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

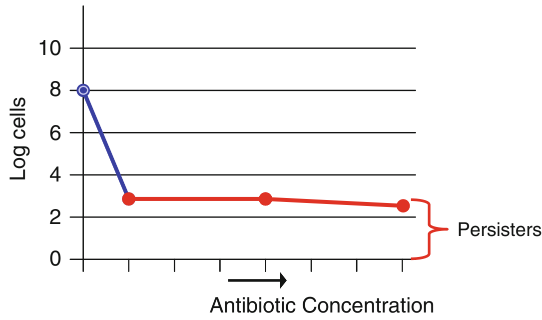


**Fig. 1** Dose-dependent killing with a bactericidal antibiotic reveals a small subpopulation of tolerant cells, persisters

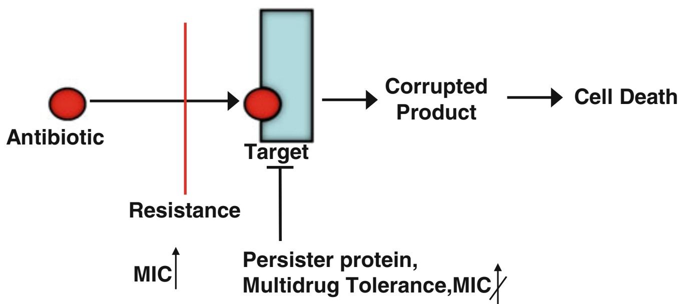


**Fig. 2** Resistance and tolerance. Bactericidal antibiotics kill cells by forcing the active target to produce corrupted products. Persister proteins act by blocking the target, so no corrupted product can be produced. By contrast, all resistance mechanisms prevent the antibiotic from binding to the target

antibiotics leads to the selection of strains that produce increased levels of persisters (Moyed and Bertrand 1983; Wolfson et al. 1990). 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 (Gibson et al. 2003). The periodic application of high doses of antibiotics provides some relief by decreasing the pathogen burden, but 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 (Smith et al. 2006). 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 (Mulcahy et al. 2010). Testing paired strains from additional patients showed that in most cases, there was a considerable increase in persister levels in the late isolate

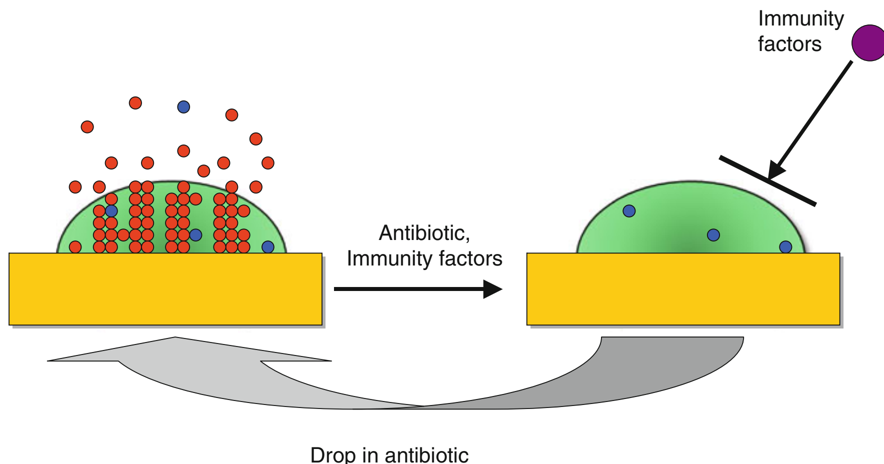


**Fig. 3** A model of relapsing biofilm infections. Regular cells (*red*) and persisters (*blue*) form in the biofilm and are shed off into surrounding tissue and bloodstream. Antibiotics kill regular cells, and the immune system eliminates escaping persisters. The matrix protects persisters from the immune system, and when the concentration of the antibiotic drops, they repopulate the biofilm, causing a relapse

from a patient. Interestingly, most of the *hip* isolates had no increase in MIC compared to 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 in 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. 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 the likely main culprit responsible for morbidity and mortality of the CF infection, it makes sense to test for their presence. Testing for persister levels is not that much more difficult as compared to 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* (Lafleur et al. 2010). These were cancer patients undergoing chemotherapy, and suppression of the immune system caused the fungal infection. In patients where the disease did not resolve, the *C. albicans* isolates were almost invariably *hip* mutants, as compared to patients where the disease cleared within 3 weeks of treatment with chlorhexidine. The eukaryotic *C. albicans* forms persisters (Al-Dhaheri and

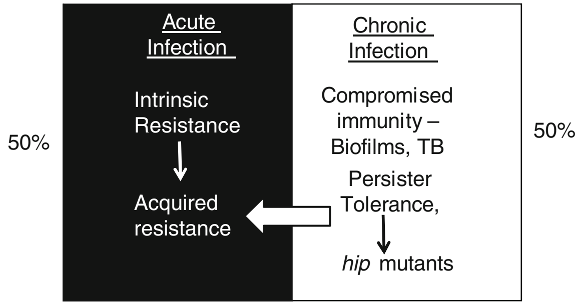


**Fig. 4** The two faces of threat. Drug resistance plays an important role in recalcitrance of acute infections, while drug tolerance is largely responsible for failures of chemotherapy in chronic infections. Tolerance allows a large population of the pathogen to linger, which increases the probability of acquiring resistance

Douglas 2008; Harrison et al. 2007; LaFleur et al. 2006) through mechanisms that are probably analogous, rather than homologous to that of their bacterial counterparts. Given the similar life styles 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 (Harrison et al. 2005a, b; Harrison et al. 2009; LaFleur et al. 2006; Spoering and Lewis 2001), allowing them to evade the immune response. However, a more general paradigm is that persisters will be 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 (Honda and Warren 2009), and the gastrointestinal tract, where a hard-to-eradicate *H. pylori* causes gastroduodenal ulcers and gastric carcinoma (Peterson et al. 2000). 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 a large reservoir of the pathogen is preserved in a dormant state in latent asymptomatic carriers (Barry et al. 2009). It is estimated that 1 in every 3 people carry latent *M. tuberculosis*, and 10% of them develop an acute infection at some stage in their lives. Virtually nothing is known about this dormant form. One simple possibility is that persisters are equivalent to the dormant form of the pathogen in a latent infection.

The above analysis underscores the significance of drug tolerance as a barrier to effective antimicrobial chemotherapy. Given its significance—roughly half of all cases of infection (Fig. 4)—the number of studies dedicated to tolerance is tiny as

compared to publications on resistance. The difficulty in pinpointing the mechanism of biofilm recalcitrance and the formidable barriers to study persister cells accounts for the lack of parity between these two comparably significant fields. Hopefully, a better balance will be achieved, and the following discussion summarizes recent advances in understanding the mechanism of tolerance.

## 2 Molecular Mechanisms of Dormancy

Persisters were initially discovered in 1944 (Bigger 1944), but the mechanism of their formation eluded us for a very long time. Only recently the molecular mechanism of dormancy began to emerge.

The most straightforward approach to finding an underlying mechanism of a complex function is by screening a library of transposon 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 (Hu and Coates 2005; Spoering 2006). With the development of the complete, ordered *E. coli* gene knockout library by the Mori group (Baba et al. 2006, the Keio collection), it seemed reasonable to revisit the screening approach. Indeed, there always remains a possibility that transposons missed a critical gene, or the library was not large enough. The use of the Keio collection largely resolves this uncertainty.

This advanced screen (Hansen et al. 2008), similarly 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 tenfold 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 (Fig. 5). The screen also produced two interesting candidate genes that may be more directly involved in persister formation—YgfA that can inhibit nucleotide synthesis, and YigB, which may block metabolism by depleting the pool of FMN.

A similar screen of a *P. aeruginosa* mutant library was recently reported (De Groote et al. 2009). 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—a single linear regulatory pathway controlling an execution mechanism. By contrast, persisters are apparently formed through a number of independent parallel mechanisms (Fig. 5). There is a considerable adaptive advantage in this redundant design—no single compound will disable persister formation.

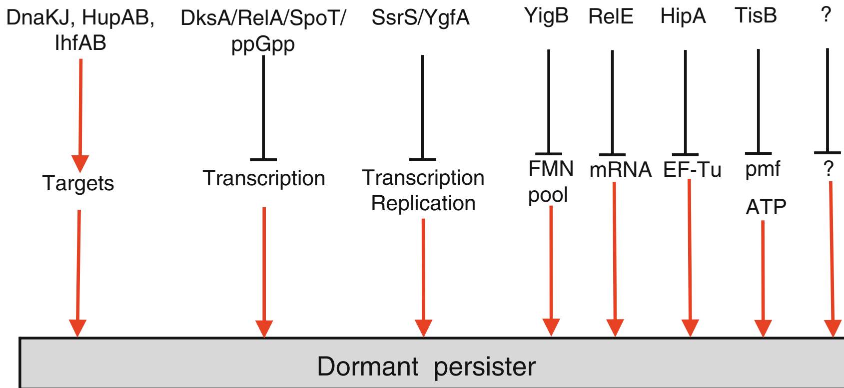


**Fig. 5** Candidate persister genes. Persisters are formed through parallel redundant pathways

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

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 (Keren et al. 2004). This allowed to isolate enough *E. coli* cells to perform a transcriptome analysis. A more advanced method aimed at isolating native persisters was developed, based on a guess that these are dormant cells with diminished protein synthesis (Shah et al. 2006). If the strain expressed degradable GFP, then cells that stochastically enter into dormancy will become dim. 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. The difference in fluorescence allowed for the sorting of the two subpopulations. The dim cells were tolerant to ofloxacin, confirming that they are persisters.

Transcriptomes obtained by both methods pointed to downregulation of biosynthesis genes, and indicated increased expression of several toxin/antitoxin modules (RelBE, MazEF, DinJYafQ, YgiU). TA modules are found on plasmids where they constitute a maintenance mechanism (Gerdes et al. 1986b; Hayes 2003). 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, while 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 and an unrelated toxin RelE induce stasis by cleaving mRNA which of course inhibits translation, a condition that can be reversed by expression of corresponding antitoxins (Christensen and Gerdes 2004; Pedersen et al. 2002). This property of toxins makes them into excellent candidates for persister genes.

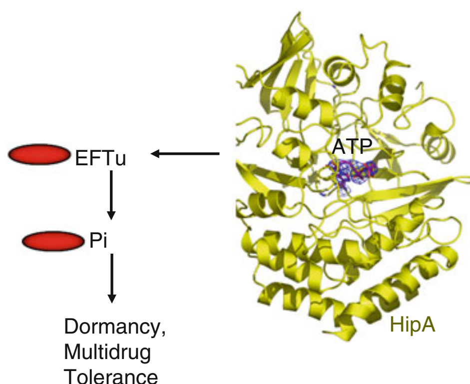


**Fig. 6** The HipA toxin causes dormancy in *E. coli* by phosphorylating elongation factor Tu which inhibits protein synthesis

Ectopic expression of RelE (Keren et al. 2004) or MazF (Vazquez-Laslop et al. 2006) strongly increased tolerance to antibiotics. The first gene linked to persisters, *hipA* (Moyed and Bertrand 1983), is also a toxin, and its ectopic expression causes multidrug tolerance as well (Correia et al. 2006; Falla and Chopra 1998). Interestingly, a bioinformatic analysis indicates that HipA is a member of the Tor family of kinases, which have been extensively studied in eukaryotes (Schmelzle and Hall 2000), 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 (Correia et al. 2006). 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 (Schumacher et al. 2009). Phosphorylated EF-Tu is inactive, which leads to a block in translation and dormancy (Fig. 6).

Deletion of potential candidates of persister genes noted above does not produce a discernible phenotype affecting persister production, possibly due to the high degree of redundancy of these elements. In *E. coli*, there are at least 15 toxin–antitoxin (TA) modules (Alix and Blanc-Potard 2009; Pandey and Gerdes 2005; Pedersen and Gerdes 1999), and more than 80 in *M. tuberculosis* (Ramage et al. 2009).

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

Several TA modules contain the Lex box and are induced by the SOS response. These are *symER, hokE, yafN/yafO* and *tisAB/istr1* (Courcelle et al. 2001; Fernandez De Henestrosa et al. 2000; Kawano et al. 2007; McKenzie et al. 2003; Motiejunaite et al. 2007; Pedersen and Gerdes 1999; Singletary et al. 2009; Vogel et al. 2004). Fluoroquinolones induce the SOS response (Phillips et al. 1987), and we tested the ability of ciprofloxacin to induce persister formation (Dörr et al. 2009, 2010).

Examination of deletion strains showed that the level of persisters dropped dramatically, 10–100 fold, in a Δ*tisAB* mutant. This suggests that TisB was responsible for the formation of the majority of persisters under conditions of

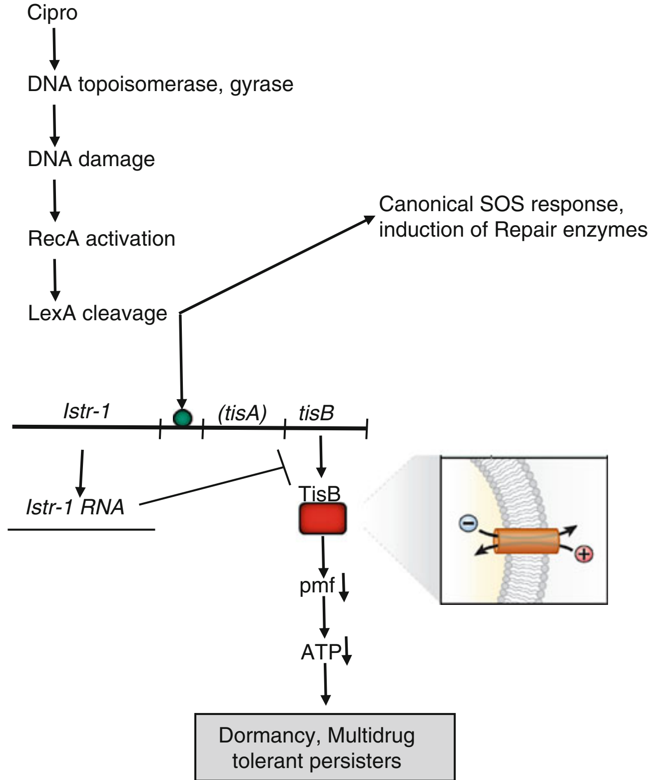


**Fig. 7** Persister induction by antibiotic. The common antibiotic ciprofloxacin causes DNA damage by converting its targets, DNA gyrase and topoisomerase, into endonucleases. This activates the canonical SOS response, leading to increased expression of DNA repair enzymes. At the same time, the LexA repressor that regulates expression of all SOS genes also controls transcription of the TisAB toxin/antitoxin module. The TisB toxin is an antimicrobial peptide, which binds to the membrane, causing an increase in pmf and ATP. This produces a systems shutdown, blocking antibiotic targets, which ensures multidrug tolerance

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 dependence on the SOS pathway.

Ectopic overexpression of *tisB* sharply increased the level of persisters. A drop in persisters in a deletion strain and increase upon overexpression gives reasonable confidence in functionality of a persister gene. The dependence of TisB-induced persisters on a particular regulatory pathway, the SOS response, further strengthens the case for TisB as a specialized persister protein (Fig. 7). Incidentally, a *tisB* mutant is not present in the otherwise fairly complete Keio knockout library, and the small ORF 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 proteins. 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 (Unoson and Wagner 2008). Bacteria, plants, and animals all produce antimicrobial membrane-acting peptides (Garcia-Olmedo et al. 1998; Sahl and Bierbaum 1998; Zasloff 2002). 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 (Gerdes et al. 1986a). High-level artificial overexpression of TisB also causes cell death (Unoson and Wagner 2008). 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.

Fluoroquinolones such as ciprofloxacin are widely used broad-spectrum antibiotics, and their ability to induce multidrug tolerant cells is unexpected and a cause of 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.

The finding of the role of TisB 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—oxidants, high temperature, low pH, membrane-acting agents. It is possible that all stress responses induce the formation of surviving persisters.

While resistance and tolerance are mechanistically distinct, there is sufficient reason to believe that tolerance may be a major cause for developing resistance. Indeed, the probability of resistance development is proportional to the size of the pathogen population, and a lingering chronic infection that cannot be eradicated due to tolerance will go on to produce resistant mutants and strains acquiring resistant determinants by transmission from other bacteria (Levin and Rozen 2006) (Fig. 4). Combating tolerance then becomes a major component in preventing resistance.

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