Multidrug Tolerance of Biofilms and Persister Cells

K. Lewis

Abstract Bacterial populations produce a small number of dormant persister cells that exhibit multidrug tolerance. All resistance mechanisms do essentially the same thing: prevent the antibiotic from hitting a target. By contrast, tolerance apparently works by shutting down the targets. Bactericidal antibiotics kill bacteria by corrupting their targets, rather than merely inhibiting them. Shutting down the targets then protects from killing. The number of persisters in a growing population of bacteria rises at mid-log and reaches a maximum of approximately 1% at stationary state. Similarly, slow-growing biofilms produce substantial numbers of persisters. The ability of a biofilm to limit the access of the immune system components, and the ability of persisters to sustain an antibiotic attack could then account for the recalcitrance of such infections in vivo and for their relapsing nature. Isolation of *Escherichia coli* persisters by lysing a population or by sorting GFP-expressing cells with diminished translation allowed to obtain a gene expression profile.

K. Lewis
Antimicrobial Discovery Center and Department of Biology, Northeastern University,
360 Huntington Avenue, Boston, MA, 02459 USA
k.lewis@neu.edu

T. Romeo (ed.), *Bacterial Biofilms.*
Current Topics in Microbiology and Immunology 322.

The profile indicated downregulated biosynthetic pathways, consistent with their dormant nature, and indicated overexpression of toxin/antitoxin (TA) modules. Stochastic overexpression of toxins that inhibit essential functions such as translation may contribute to persister formation. Ectopic expression of RelE, MazF, and HipA toxins produced multidrug tolerant cells. Apart from TA modules, *glpD* and *plsB* were identified as potential persister genes by overexpression cloning of a genomic library and selection for antibiotic tolerance. Yeast *Candida albicans* forms recalcitrant biofilm infections that are tolerant to antibiotics, similarly to bacterial biofilms. *C. albicans* biofilms produce multidrug tolerant persisters that are not mutants, but rather phenotypic variants of the wild type. Unlike bacterial persisters, however, *C. albicans* persisters were only observed in a biofilm, but not in a planktonic stationary population. Identification of persister genes opens the way to a rational design of anti-biofilm therapy. Combination of a conventional antibiotic with a compound inhibiting persister formation or maintenance may produce an effective therapeutic. Other approaches to the problem include sterile-surface materials, prodrug antibiotics, and cyclical application of conventional antimicrobials.

1 Biofilms and Persisters

According to the CDC, 65% of all infections in developed countries are caused by biofilms, bacterial communities that settle on a surface and are covered by an exopolymer matrix (Hall-Stoodley et al. 2004). These include common diseases such as childhood middle ear infection and gingivitis; infections of all known indwelling devices such as catheters, orthopedic prostheses, and heart valves; and the incurable disease of cystic fibrosis. Biofilms are produced by most if not all pathogens. *Pseudomonas aeruginosa*, causing an incurable infection in cystic fibrosis patients (Singh et al. 2000), and *Staphylococcus aureus* and *Staphylococcus epidermidis*, infecting indwelling devices (Mack et al. 2004), are probably the best-known biofilm-producing organisms. Biofilm infections are highly recalcitrant to antibiotic treatment. However, planktonic cells derived from these biofilms are in most cases fully susceptible to antibiotics. Importantly, biofilms do not actually grow in the presence of elevated levels of antibiotics, meaning they do not exhibit increased resistance as compared to planktonic cells (Lewis 2001b). But if biofilms are not resistant, how do they resist being killed? Biofilm resistance to killing 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 (Brooun et al. 2000; Lewis 2001b; Spoering and Lewis 2001).

Most of the cells in a biofilm are actually highly susceptible to a bactericidal agent such as a fluoroquinolone antibiotic or metal oxyanions that can kill both rapidly dividing and slow-growing or nongrowing cells (Spoering and Lewis 2001; Harrison et al. 2005a, 2005b) (Fig. 1). This is important, since cells in the biofilm are slow growing, and many are probably in stationary state. The experiment also revealed a small subpopulation of cells that remain alive irrespective of the

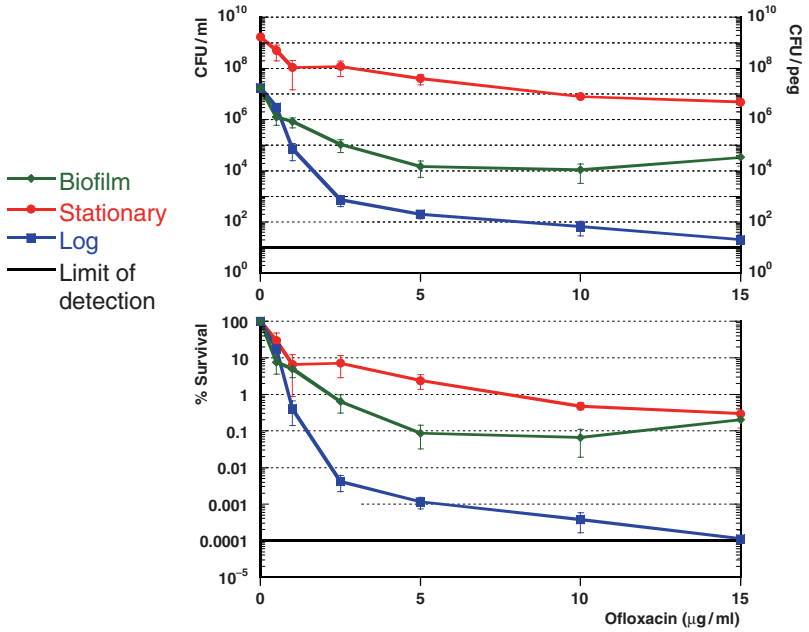

Fig. 1 Killing of logarithmic-phase, stationary-phase, and biofilm cultures of *P. aeruginosa* by ofloxacin. *Upper panel*, the concentration of cells for planktonic cultures is given in the left y axis, and the amount of cells released from a peg carrying a biofilm is given in the right y axis. The estimate of the concentration of cells in the biofilm is 10^{11}/ml of biofilm. *Lower panel*, results of the upper panel are recalculated as % survival. The limit of detection is indicated by the solid horizontal line

concentration of the antibiotic. Note that the cell concentration in log, stationary, and biofilm cultures is obviously different. Taking into account the thickness of the biofilm on the peg (*), its dimensions and the total number of cells per peg, we estimate the density of cells in the biofilm to be 10^{11}, substantially higher than in the planktonic cultures. In each case, a persister plateau is produced, meaning that starting at a certain level, further increase in the antibiotic concentration does not result in additional killing. This obviates such possible problems as diminishing the concentration of the available antibiotic by cell binding.

The level of these surviving persisters was even greater in the nongrowing stationary population. In a test tube, a stationary culture appears more tolerant than the biofilm. However, this situation is likely reversed in vivo. Antibiotic treatment will eliminate the bulk of both biofilm and planktonic cells, leaving intact persisters. At this point, the similarity with an in vitro experiment probably ends. The immune system will be able to mop up remaining planktonic persisters, just as it eliminates nongrowing cells of a population treated with a bacteriostatic antibiotic (Fig. 2). However, the biofilm matrix protects against immune cells (Leid et al. 2002; Jesaitis et al. 2003; Vuong et al. 2004), and its persisters will survive. After

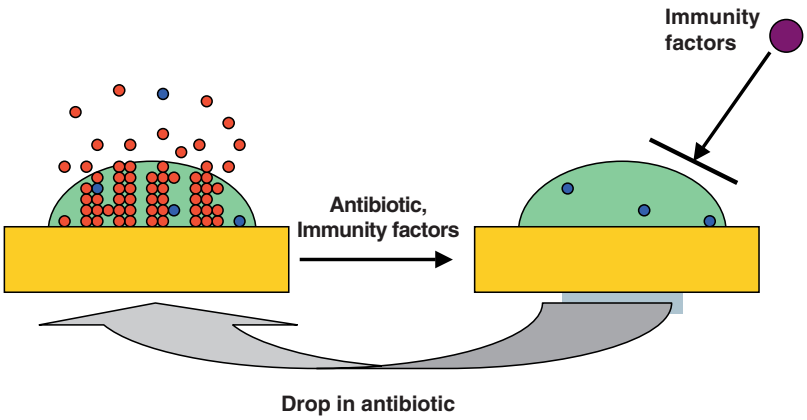

Fig. 2 Model of biofilm resistance based on persister survival. An initial treatment with antibiotic kills planktonic cells and the majority of biofilm cells. The immune system kills planktonic persisters, but the biofilm persister cells are protected from host defenses by the exopolysaccharide matrix. After the antibiotic concentration drops, persisters resurrect the biofilm and the infection relapses

antibiotic concentration drops, persisters will repopulate the biofilm, which will shed off new planktonic cells, producing the relapsing biofilm infection (Lewis 2001b). The problem of biofilm resistance to "everything" largely defaults to understanding persisters.

Persisters were described by Joseph Bigger in 1944 in one of the first studies of penicillin action (Bigger 1944). Bigger discovered that penicillin lysed a growing population of *Staphylococcus*, but plating this transparent solution on nutrient medium produced surviving colonies. In order to test whether these were mutants, the colonies were grown, treated with penicillin, and the new population again produced a small number of persisters surviving lysis. This experiment was repeated recently with *Escherichia coli* and several different antibiotics and produced similar results (Keren et al. 2004a; Wiuff et al. 2005). By the time the mechanism of biofilm resistance to killing was being investigated, Bigger's work was all but forgotten, a curiosity known to few microbiologists. Harris Moyed picked up the problem in the 1980s and undertook a targeted search for persister genes (Moyed and Bertrand 1983; Moyed and Broderick 1986; Scherrer and Moyed 1988; Black et al. 1991, 1994). He reasoned that treating a population of *E. coli* with ampicillin would select for mutants with increased production of persisters. After ampicillin application, cells were allowed to recover, and the enrichment process was repeated. This is different from the conventional selection for resistant mutants, where cells that can grow in the presence of antibiotic are favored. After testing for mutants that had the same MIC to ampicillin (thus not resistant), but survived better, several strains were obtained, and one of them was used to map the mutation to a *hipBA* locus. The mutant appeared to carry a mutation in the *hipA* gene, and this *hipA7* allelic strain was found to make 1% persisters in exponential cultures, about 1,000 times more

than the wild type. Deletion of *hipBA* had no apparent effect on persister formation, suggesting that *hipA7* mutant (Korch et al. 2003) produced a pleiotropic artifact. Another possibility is that *hipA* is part of a redundant set of genes, and knocking out any single one does not produce a phenotype. Like Bigger's work before him, the studies of Moyed were largely forgotten.

The finding of persisters in biofilms rekindled an interest in this curiosity, which appears to be responsible for a major part of recalcitrant human infectious diseases. We will summarize here what we currently know about the biology of persisters. But first, let us consider the difference between resistance of regular cells and drug tolerance of persisters. This will provide a useful framework for the subsequent discussion of persisters and their properties.

1.1 Multidrug Resistance and Multidrug Tolerance, Two Mechanistically Distinct Menaces

Numerous mechanisms of drug resistance have been described, and in most cases we have a fairly 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; antibiotic efflux; and restricted antibiotic permeation (Lewis et al. 2002; Levy and Marshall 2004). It is interesting to note that all theoretically logical possibilities of antibiotic resistance seem to have been realized in nature. Importantly, all of these mechanisms do essentially the same thing: prevent the antibiotic from binding to the target (Fig. 3). Each of these resistance mechanisms allows cells to grow at an elevated level of antibiotic. It is important to note that bactericidal antibiotics kill the cell not by merely inhibiting

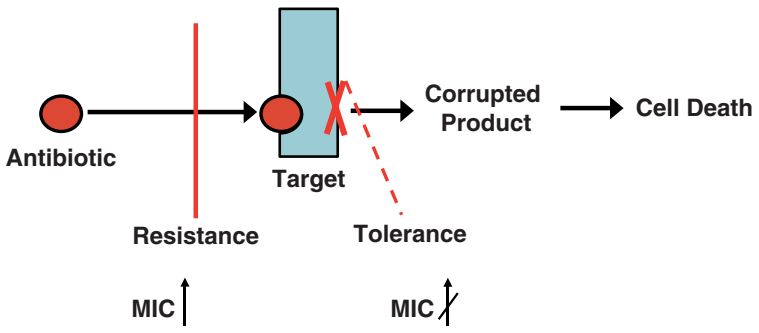

Fig. 3 Antibiotic resistance vs tolerance. Resistance mechanisms prevent an antibiotic from binding to the target, which leads to an increase in the MIC. Bactericidal antibiotics act by corrupting the target, producing a toxic product that kills the cell. Tolerance occurs when a persister protein blocks the target, preventing formation of a toxic product

the target, but by corrupting its function in a manner that creates a toxic product. Aminoglycoside antibiotics kill the cell by interrupting translation, which creates misfolded toxic peptides (Davis et al. 1986). β-Lactam antibiotics such as penicillin inhibit peptidoglycan synthesis, which activates, by a largely unknown mechanism, autolysin enzymes present in the cell wall (Bayles 2000). 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 enzyme converts into an endonuclease (Hooper 2002).

We think that tolerance acts by a preventative blocking of the antibiotic targets (Fig. 3). If persisters are dormant and have little cell wall synthesis, translation or topoisomerase activity, then the antibiotics will bind to their targets, but will be unable to corrupt them. In this manner, tolerance does provide resistance to killing from everything, but at a price of nonproliferation.

The simplest way to form a dormant persister could be through fluctuations in the levels of a potentially large number of proteins that upon overproduction become toxic to the cell and stop growth. This possibility has been elegantly demonstrated by expressing in *E. coli* proteins that are known to inhibit growth when overproduced (Vazquez-Laslop et al. 2006). Cells overexpressing the chaperone DinJ or the *Salmonella typhimurium* PmrC, an enzyme that transfers phosphoethanolamine to lipid A, stopped growing and became highly tolerant to ampicillin and ciprofloxacin. However, it does not seem that this simple mechanism is primarily responsible for making persisters in the wild type, at least not in *E. coli*. Examination of the rate of *E. coli* persister formation over time showed very few of them in early exponential state, followed by a sharp rise in mid-exponential, and reaching about 1% in nongrowing stationary state. In order to learn whether persisters in early exponential were leftovers from stationary state, or formed de novo, the culture was kept at this stage by repeated reinoculation (Keren et al. 2004a). After four reinoculations from early exponential to early exponential, persisters completely disappeared. This simple experiment essentially rules out nonspecific mechanisms of persister formation. Indeed, mistakes are unavoidable and should happen at early exponential state as well. This experiment also indicates that persisters are preformed, rather than being produced in response to antibiotics.

Persister rise at mid-exponential state has been observed in several species, but its nature remains unknown. Quorum sensing does not seem to play a role, since addition of spent medium to early exponential cultures of *E. coli* or *P. aeruginosa* did not appreciably increase the persister level (K. Lewis, unpublished data). Whatever the mechanism of the rise, the dynamics of persister formation provide an interesting insight into the strategy of persistence. Persisters are cells that temporarily forfeit propagation in favor of survival. Their strategy is distinct from the well-studied stress responses, when the entire population expresses resistance proteins (such as heat shock or SOS) in response to a nonlethal dose of a deleterious factor. Persisters are able to survive a dose of antibiotic that kills regular cells. However, persisters only become prominent in a fairly dense cell population. This strongly suggests that persisters are essentially altruistic cells, which ensure survival of a kin population in the presence of a lethal factor. In early exponential

phase, there are essentially no kin (no means for a cell to distinguish between few vs no neighbors), and no one to benefit from an act of altruism. The highest level of persisters reached in a stationary population suggests that their main function is actually ensuring survival of this nongrowing population. But since persisters are also nongrowing, why does the entire stationary population not enter into this protected state? The benefit of being a regular cell apparently stems from the ability to rapidly resume growth, which may be more problematic for a dormant persister. Since the majority of cells in an *E. coli* or *P. aeruginosa* stationary population are regular cells, this suggests that the optimal individual strategy is not to enter into persistence, again suggesting that the persister state is an altruistic behavior benefiting the kin.

The stationary state experiment also provides an important distinction between merely not growing and persistence. Some antibiotics, like β-lactams and aminoglycosides, strongly depend on cell growth for their action, and a nongrowing stationary population is indeed tolerant to these compounds. Fluoroquinolones and mitomycin C, for example, can kill nongrowing cells, and treatment with these compounds reveals a small subpopulation of tolerant persisters in the stationary state.

1.2 In Search of the Mechanism of Persister Formation

Identification of the mechanism of persister formation presents a formidable challenge due to an apparent redundancy of persister genes. Thus, attempts to identify persister genes by screening transposon insertion libraries for either increased or decreased survival to antibiotics were not successful (Hu and Coates 2005; Spoering et al. 2006). In a recent report, phoU was identified as a putative persister gene using a similar approach. However, the *phoU* mutant had a decreased MIC to a number of antibiotics. This suggests that *phoU*::Tn is a pleiotropic mutation. It is interesting to compare this experience with identifying genes controlling another function that produces dormant cells: sporulation. It is easy to obtain specific *spo* mutants specifically lacking the ability to make spores from a knockout library (*), and this is indeed how most *spo* genes were identified. Genes controlling tolerance resemble in this regard those coding for multidrug resistance pumps (MDRs). In *P. aeruginosa*, for example, there are at least 13 RND family MDRs, but knockout out of most of them does not produce a discernible phenotype (*). At the same time, overexpressing any single MDR produces multidrug resistance. We therefore reasoned that persister genes may be identified by screening or selecting a library cloned into an expression vector for gain of function. In this case, even a weak contributor to a multigene function can be identified when overexpressed. However, this approach is problematic as well, since overproduction of many proteins leads to misfolded toxic products that can stop cell growth and will create an artifact emulating a dormant state, as discussed above. It appears that standard approaches of molecular genetics are poorly suited to search for persister genes, which probably explains the slow pace of discovery in this area.

Another barrier to discovery has been a lack of approaches to isolate persister cells. The first method to isolate persisters was recently reported, based on simply sedimenting surviving cells from a culture lysed by ampicillin (Keren et al. 2004b). This method has its limitations: it requires a rapidly growing culture for ampicillin to lyse it, and the fraction of persisters in such a population is small, approximately 10^{-5}. In *E. coli*, this necessitated the use of a *hipA7* strain overproducing persisters. In addition, these persisters are exposed to an antibiotic. These limitations notwithstanding, enough cells were collected to obtain a gene expression profile. The profile showed downregulation of proteins involved in energy production and nonessential functions such as flagellar synthesis, suggesting that persisters are dormant cells. This is consistent with the finding that persisters formed by a *hipA7* (high persistence) strain of *E. coli* are nongrowing (or slow growing) cells (Balaban et al. 2004). The profile also pointed to proteins that may be responsible for dormancy: RMF, a stationary state inhibitor of translation (Yoshida et al. 2002), SulA, an inhibitor of septation (Walker 1996), and toxin-antitoxin (TA) module elements RelBE, DinJ, and MazEF (Christensen and Gerdes 2003; Christensen et al. 2003). Homologs of TA modules are found on plasmids where they constitute a maintenance mechanism (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. MazEF was proposed to serve as a programmed cell death mechanism (Sat et al. 2001). However, it was reported recently that MazF and an unrelated toxin RelE do not actually kill cells, but induce stasis by inhibiting translation, a condition that can be reversed by expression of corresponding antitoxins (Pedersen et al. 2002; Christensen et al. 2003).

Expression of RelE, a toxin that causes reversible stasis by inhibiting cleaving mRNA and inhibiting translation, strongly increased tolerance to antibiotics (Keren et al. 2004b). Expression of a toxin HipA increased tolerance as well (Falla and Chopra 1998; Correia et al. 2006; Korch and Hill 2006; Vazquez-Laslop et al. 2006). Interestingly, a bioinformatics 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 $Mg2^{+}$-binding sites abolishes its ability to stop cell growth and confer drug tolerance (Correia et al. 2006). Knowing that HipA is a kinase provides an additional tool to search for the target, which is yet to be identified.

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 ten toxin-antitoxin (TA) modules, and more than 60 in *Mycobacterium tuberculosis* (Gerdes et al. 2005).

Several independent lines of evidence point to persister dormancy: lack of growth in the presence of antibiotics (by contrast to resistant mutants), downregulation of biosynthetic pathways, and an elegant demonstration of slow growth or no growth in persisters formed by the *E. coli hipA7* strain (Balaban et al. 2004). In the latter study, cells were placed in troughs of a multichannel chip that restricts mobility and makes it possible to simultaneously videotape growth and division of many individual cells in the channels. The device also made it possible to flush the medium, and application of ampicillin caused lysis of cells. However, cells that did not lyse were those that had little growth preceding the application of ampicillin.

Based on these data, we reasoned that dormancy may be used to physically sort naïve persister cells from a wild type population (Shah et al. 2006). Dormancy implies low levels of translation, which can then enable differential sorting based on expression of a detectable protein. In *E. coli* ASV, a degradable GFP is inserted into the chromosome in the λ attachment site and expressed from the ribosomal *rrnB*P1 promoter, the activity of which is proportional to the rate of cell growth (Fig. 4). The half-life of degradable GFP is less than 1 h, and it should be effectively cleared from dormant cells. This would then enable sorting of dim persister cells. A logarithmically growing population of *E. coli* ASV was sorted with a high-speed cell-sorter using forward light scatter, which detects particles based on size. This enabled detection of cells irrespective of their level of fluorescence. Sorting by fluorescence showed that the population consisted of two strikingly different types of cells: a bright majority and a small subpopulation of cells with no detectable fluorescence (Fig. 4). Fluorescent microscopy confirmed that the sorted bright cells were indeed bright green, while the dim ones had no detectable fluorescence. The dim cells were also smaller than the fluorescent cells, and in this regard resembled stationary state cells. Sorted dim cells were exposed to a high level of ofloxacin that rapidly kills both growing and nongrowing normal cells, but has no effect on persisters. The majority of this subpopulation survived, as compared to a drastic drop in viability of the sorted bright cells. This experiment showed that the sorted dim cells are dormant persisters.

The sorting method provides a general approach to obtaining naïve persisters from a wild type population of any species. These cells can then be used to obtain an expression profile and to study a variety of functions by biochemical methods. However, there is still room for improvement: sorting is relatively slow, costly, and results in a limited amount of material that precludes proteome analysis, for example. A rapid method for obtaining large quantities of persisters has yet to be developed.

Knowing that persisters are dormant cells bolsters the case for TA module involvement in persister formation. Indeed, TAs seem to be ideally suited for the task. Reversible action of toxins such as RelE and MazF, inhibition of important cellular functions by toxins capable of creating a dormant state, and the presence of TA modules in the chromosomes of all known free-living bacteria makes them attractive candidates for persister genes.

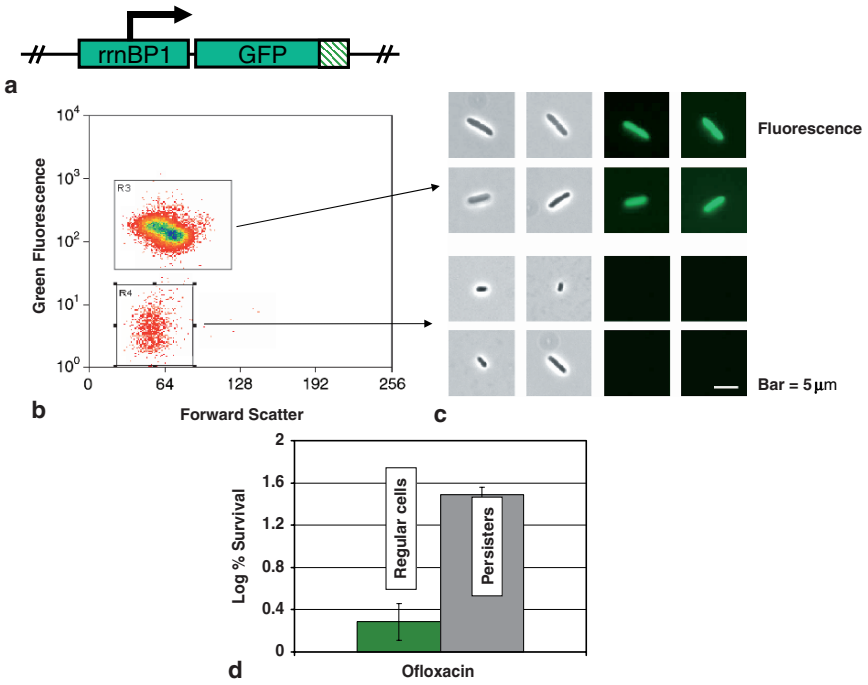

Fig. 4 Sorting of *E. coli* persister cells from a growing population. **a** Graphical representation of the reporter. An unstable variant of GFP was placed downstream of a ribosomal promoter, *rrnB*P1. **b** *E. coli* ASV cells containing the degradable rrnBP1GFP reporter cassette were grown in LB medium to mid-exponential phase (~1×10^8 cells/ml) at 37°C with aeration and sorted. Two populations were detected using forward light-scatter, one that fluoresced brightly (R3), and another that did not (R4). **c** The sorted populations were visualized by epifluorescent microscopy (bar, 5 µm). **d** Cells were sorted as described in (**a-c**). Once sorted, both populations were treated with ofloxacin (5 µg/ml) for 3 h, diluted and spotted onto LB agar plates for colony counts. Cells carrying a degradable GFP under the control of a ribosomal promoter whose activity is proportional to the rate of growth were sorted, and the dim and bright cells were then exposed to an antibiotic to test their tolerance

1.3 A Generalized Hunt for Persister Genes

Expression cloning could in principle reveal even minor participants that contribute to persister formation. The problem with this approach, however, is that overexpression of many proteins causes nonspecific toxicity that stops cell growth and appears as natural dormancy. In order to make this approach work, we decided to use mild overexpression by cloning an *E. coli* library into a low-copy vector, using native promoters for expression, and introducing a growth step between rounds of selection by a bactericidal antibiotic (Spoering et al. 2006). In this manner, we could

select against any cells that grew considerably slower than the wild type. This procedure enriched the population in cells with elevated persister production. A clone with consistent persister overproduction was sequenced and appeared to carry the *glpD* gene coding for glycerol-3-phosphate dehydrogenase. A knockout of *glpD* caused a modest decrease in persister levels in a stationary (but not exponential) culture. Interestingly, there is a second glycerol-3-phosphate dehydrogenase in *E. coli*, GlpA and a double *glpD,glpA* knockout had a considerably stronger phenotype as compared to *glpD*. This explains why we (and others) missed *glpD* in a screen of a knockout library for persister genes, and reinforces the idea of persisters being controlled by functionally redundant genes.

The opposing behavior of an overexpression vs a knockout confirms participation of GlpD in persister formation. The mechanism by which GlpD affects persistence is currently under investigation. Testing mutants affected in proteins within the GlpD metabolic network indicated another interesting component that affects persistence: PlsB, a well-conserved bacterial protein. The PlsB enzyme uses G3P to produce 1-acyl-G3P and is essential, preventing straightforward analysis of a null mutant. However, a strain with a mutant PlsB enzyme with a higher Km has been described (*plsB26*; Heath and Rock 1999), and was used to examine persister formation. The *plsB26* strain grew normally and showed the same MIC to ampicillin and ofloxacin as an isogenic wild type control. Production of persisters by the *plsB26* strain in a stationary culture was 100-1,000 times lower than the wild type, suggesting that PlsB may be a useful target for anti-persister therapy. PlsB is an abundant housekeeping protein, and its level did not change in the transcriptomes of purified persisters. It seems that PlsB, rather than being involved in persister formation, is a persister maintenance gene. It appears that in order to survive, persisters depend on the ability to maintain their membrane integrity, which requires PlsB-dependent phospholipid synthesis.

The application of expression cloning seems promising and suggests that it will yield additional persister genes and should work for any bacterial species.

The multitude of proteins that can induce multidrug tolerance is reminiscent of the many MDR pumps responsible for multidrug resistance. It appears that microbial populations have evolved two complementary and highly redundant strategies to protect themselves from antimicrobials: multidrug efflux, and when this fails, multidrug tolerance of persister cells.

1.4 Persisters and Stochastic Phenomena

Persisters make up a small subpopulation, and in mid-log phase *E. coli* produces as little as 10^{-5} surviving tolerant cells. Given that all of the cells in a population are genetically identical kin, it seems that persisters have to be produced by a stochastic process (Lewis 2000; Keren et al. 2004b; Lewis et al. 2005). Indeed, what would be the alternative? Fluctuations in the levels of a small number of

dedicated proteins are probably responsible for persister formation. The absence of persisters in an early exponential population could then be due to low overall levels of persister proteins at this stage. Note that persister proteins HipA and GlpD only have an effect in stationary state, suggesting that their levels do not undergo large fluctuations during exponential growth. Indeed, both HipA and GlpD are capable of producing persisters in an exponential population upon artificial overexpression, and the lack of a phenotype for a deletion mutant at this growth phase strongly suggests that they are not reaching high enough levels in rapidly growing cells. It seems that two processes control persister formation: a stochastic fluctuation in the level of persister proteins and a controlled, regulated mean level of expression of these proteins, which is dependent on the density of the population. The fluctuation happening around an elevated base will then allow persister proteins to reach a level of expression sufficient to produce persisters in a dense population.

Stochastic phenomena have been described for a large number of functions, both in bacteria (Avery 2006; Dubnau and Losick 2006) and in eukaryotes (Kaern et al. 2005). In bacteria, stochastic processes are responsible for determining the part of the population that enters sporulation in *Bacillus subtilis* (Chung et al. 1994), spontaneous SOS induction in *E. coli* (McCool et al. 2004), or cannibalism in *B. subtilis* (Gonzalez-Pastor et al. 2003). The most visible case of bacterial decision making is chemotaxis, which relies on a trial-and-error random walk (Berg and Brown 1972). Counterclockwise rotation of a bacterial flagellum produces a bundle that propels the cell forward, but periodically a switch changes it to counterclockwise, the bundle falls apart, and the cell tumbles before the next run resumes in a new direction. 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 (Spudich and Koshland 1976). Apparently, random changes in the expression of chemotaxis proteins produce another level of stochastic variation of individuality in a population. Two particular proteins, CheZ and CheY, of the many elements involved in the chemotaxis signal transduction appeared to be responsible for this noise generation (Korobkova et al. 2004).

The reason bacteria use stochastic processes in decision making is probably similar to our flipping a coin when a problem does not have a unique logical solution. The bacterial cell is too small to have a significant measurable difference between concentrations of a chemoeffector along its length, and trial-end-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 will induce a dormant state.

There is another level of noise in persister formation that we do not currently understand. This is the distinct variation in persister levels among populations grown under seemingly identical conditions in parallel test tubes or wells of a microtiter plate ((Wiuff et al. 2005); 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.

1.5 Is Multidrug Tolerance Transmissible?

Drug resistance is highly transmissible. Virtually every mechanism of resistance we know of can be present on a plasmid or a transposon. Transmissibility enables migration of resistance genes from unknown soil microorganisms into human pathogens, which is largely responsible for the increasing failure of once effective antibiotic therapies. One may suggest by simple analogy that multidrug tolerance may be transmissible as well. However, MDT mechanisms are universally present in bacteria, since all tested species make persisters, and there does not seem to be a need for transmission. At the same time, several observations point to possible transmission of drug tolerance.

As we have noted in a previous section, TA modules were first discovered as a plasmid maintenance mechanism (Gerdes et al. 1986). The toxins may 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 translational inhibitors RelE and MazF (Gerdes et al. 2005). Interestingly, the killer toxins improve plasmid maintenance 100- to 1,000-fold, while stasis toxins improve plasmid maintenance by only four- to tenfold. The obvious question is why has an ineffective maintenance mechanism been recruited, while an effective one is available and may easily be borrowed from another plasmid by horizontal transmission. It may be that the stasis toxins justify their presence by increasing the host's multidrug tolerance.

An interesting case of possible transmissible MDT is the presence of a HipBA homolog on an R391 transposon-like IncJ element found in Gram-negative bacteria (Boltner et al. 2002). The element adds an additional copy of the specialized MDT gene *hipA*, which may increase the level of persister production.

Finally, there is an unusual case of transmissible resistance that appears more like tolerance. The plasmid-coded Qnr proteins confer relatively low (but clinically significant) resistance to fluoroquinolones in Gram-negative species (Tran and Jacoby 2002; Vetting et al. 2006). The crystal structure of a Qnr homolog, the chromosomally coded MfpA of *M. tuberculosis* has been reported. Remarkably, MfpA is a structural DNA mimic that binds DNA gyrase, the target of fluoroquinolones (Hegde et al. 2005). The pentapeptide repeat in the Qnr family proteins produces a helical structure peculiarly resembling the double helix of DNA. MfpA binds to and sequesters the gyrase, inhibiting the enzyme and preventing the gyrase/fluoroquinolone complex from nicking the DNA. It would be interesting to see how much tolerance Qnr and MfpA add to the cell: how many more persisters will survive treatment with a fluoroquinolone?

1.6 Persisters in Yeast Biofilms

Eukaryotic yeasts have a lifestyle that is very similar to that of prokaryotic microorganisms. Not surprisingly, analogous adaptations evolved in these two groups in response to similar environmental challenges through convergent evolution. For example, yeasts form biofilms that, similarly to bacterial biofilms, are responsible for highly recalcitrant infections (Kumamoto and Vinces 2005).

The focus of yeast biofilm research has been on *C. albicans*, an important human pathogen that causes oral thrush, relapsing vaginosis, and is a leading cause of morbidity and mortality in immunocompromised individuals. The biofilm forms when single cells attach to a surface and grow into microcolonies, which then merge and produce a complex 3D structure that is held together by hyphae and an exopolymer matrix (Chandra et al. 2001). The biofilm contains a mixture of yeast, hyphae, and pseudohyphae. Similarly to bacteria, yeast biofilm exopolymer matrix restricts penetration of immune system components (Hoyle et al. 1990; von Eiff et al. 1999), but does not appreciably hinder diffusion of antifungal drugs (Baillie and Douglas 2000; Samaranayake et al. 2005).

Genes encoding multidrug resistance (MDR) pumps MDR1, CDR1, and CDR2 are upregulated upon attachment of *C. albicans* cells to a surface, and this accounts for the resistance of young biofilms to azole antibiotics (Mukherjee et al. 2003). However, the high level of drug resistance of mature biofilms (≥48 h) was not affected by deletion of all three of these genes either singly or in combination, including an *mdr1Δ cdr1Δ cdr2Δ* triple mutant (Ramage et al. 2002; Mukherjee et al. 2003; Kumamoto 2005). Decreased ergosterol content (Mukherjee et al. 2003; Kumamoto 2005) and a diminished level of ergosterol biosynthetic gene expression (Garcia-Sanchez et al. 2004) have been reported in mature *Candida* biofilms and may contribute to drug resistance. Indeed, azoles act by inhibiting ergosterol biosynthesis, and amphotericin B binds to ergosterol. However, ergosterol is unlikely to be involved in the action of echinocandins that inhibit the synthesis of cell wall B-glucan (Datry and Bart-Delabesse 2006), or chlorhexidine, a membrane-active antiseptic that is very effective against bacteria that lack sterols. In essence, the mechanism of *C. albicans* biofilm antifungal resistance remains largely unknown.

We recently examined biofilm resistance of *C. albicans*, following the same approaches used previously for bacteria. A dose-dependent experiment with two highly microbicidal agents, amphotericin B and chlorhexidine (the only compounds that can kill nongrowing yeast cells) showed complete elimination of cells in an exponential and stationary planktonic populations. However, a distinctly biphasic killing was observed in a mature biofilm, indicating the presence of persisters (LaFleur et al. 2006). Similarly to bacteria, yeast persisters are not mutants: upon reinoculation, surviving cells reproduced the original wild type population with a new fraction of persister cells. Staining with fluorescein, which specifically binds to dead yeast cells, showed live persisters within a yeast biofilm treated with amphotericin. These rare live cells were either yeast or pseudohyphae and were

morphologically unremarkable. Sorting this stained population showed that dim cells form colonies, while bright ones do not. This method, similarly to the approach described for bacteria, opens the way for obtaining a gene expression profile of yeast persisters.

Quite unexpectedly, *C. albicans* persisters were only apparent in a biofilm culture, and not in a nongrowing stationary population. Both biofilm and planktonic stationary cultures produce a mix of cell types, including yeast, pseudohyphae, and hyphae. The specific production of persisters in a biofilm is distinctly different from what is observed in bacteria, where a stationary planktonic culture actually makes more persisters than a biofilm (Spoering and Lewis 2001). This probably suggests that the biofilm, and not the planktonic population is the survival mode of yeast life, and that is where specialized survivor cells are produced.

Dependence of persister production on biofilm formation suggested that these two forms of yeast populations may share part of the same developmental program. A number of genes involved in yeast biofilm formation have been identified, and mutants deleted in these elements were tested for persister production (LaFleur et al. 2006). Surprisingly, all tested mutants appeared to produce normal levels of persisters. Among the tested mutants was the flo8 strain, which does not make hyphae, and its biofilm consists of a simple layer of yeast cells attached to the surface (Cao et al. 2006). This indicates that attachment is sufficient for persister formation.

A surface contact-dependent Mck1p kinase that affects biofilm formation and invasiveness in *C. albicans* was recently described (Kumamoto 2005), but a strain deleted in mck1 was able to produce normal levels of persisters as well. Our analysis of biofilm mutants suggests that known genes are not involved in persister formation. Persister isolation based on cell sorting opens the possibility of obtaining their transcription profile, which is likely to point to persister genes.

2 In Search of Therapy: Persister Eradication

Given the prominent role of tolerance in infectious disease, the need for compounds that could eradicate persisters is obvious. Another important factor to consider is the potential causality between tolerance and the rise of resistance. A lengthy, lingering infection that is not eradicated due to its tolerance is likely to provide a fertile ground for producing resistant mutants, or for acquisition of resistance determinants through horizontal transmission from other species. A mathematical model predicts that tolerance substantially increases the danger of resistance (Levin and Rozen 2006). This observation provides an additional incentive for developing compounds that are able to sterilize, rather than merely suppress an infection.

Before we describe possible approaches to eradicating persisters, let us put the problem into perspective and consider the state of affairs in the general field of anti-infective drug discovery. This has been described as a crisis due to

an innovation gap (Walsh 2003b). Forty years separate the discovery of the last major class, fluoroquinolones, from the recently introduced linezolid, a narrow-spectrum protein synthesis inhibitor acting against Gram-positive bacteria (Zurenko et al. 1997). During this time, pathogens did not take a break, but rapidly acquired resistance to all known antibiotics. There have been several recent successes in addition to linezolid, which include daptomycin (Cubicin, Cubist), a natural product lipopeptide, ramoplanin, a natural product depsipeptide (Genome Therapeutics, currently Oscient), and dalbavancin (Vicuron, currently Pfizer), a molecule related to vancomycin, all acting against Gram-positive species. The recently approved tigecycline (Tigacyl, Wyeth) is a fairly broad-spectrum compound loosely based on tetracycline that is not subject to existing resistances toward the parent antibiotic.

The crisis nonetheless looms, caused by a conspicuous lack of novel classes of broad-spectrum antibiotics in the face of growing drug resistance (Dougherty et al. 2002; Boggs and Miller 2004; Bush 2004; Clardy and Walsh 2004; Projan and Shlaes 2004; Schmid 2004; Silver 2006). The reasons for the crisis are well understood. Bacteria and fungi were the source of natural products, including broad-spectrum compounds, that fueled the golden era of antibiotic discovery during the 1940s and 1950s; Schatz et al. 1944; Demain and Fang 2000; Walsh 2003a). However, culturable microorganisms on which anti-infective drug discovery has been based make up only 0.01%-1% of the total diversity in the environment (Osburne et al. 2000), and this limited resource became overmined.

Synthetic approaches were unable to replace natural products in developing new anti-infective therapies. While it is certainly possible to find synthetic inhibitors against defined bacterial protein targets by screening compound libraries in vitro, attempts to convert such hits into broad-spectrum leads using medicinal chemistry have not been successful. The main obstacle to development of broad-spectrum antibiotics is the penetration barrier of the cell envelope of Gram-negative bacteria. The barrier is multilayered and consists of an inner membrane that restricts the passage of hydrophilic compounds, an outer membrane that restricts penetration of amphipathic compounds (which essentially all drugs are), and MDR pumps that efflux amphipathic substances across the outer membrane. In short, the envelope is designed in a way to restrict penetration of *all* molecules, while nutrients enter through porins and specialized transporters (Lewis and Lomovskaya 2002; Li and Nikaido 2004). We do not have a rational approach to impart a drug candidate with an ability to cross this barrier, and trial-and-error synthesis does not produce permeating compounds with a measurable degree of probability. As a result, most companies have focused on development of narrow-spectrum antibiotics, or on compounds that are modifications of existing classes. We find ourselves increasingly vulnerable to multidrug-resistant Gram-negative pathogens (Meyer 2005).

Some encouraging developments in solving the antibiotic crisis are worth mentioning. Developing MDR inhibitors is an attractive strategy to produce broad-spectrum dual-compound therapies (Markham and Neyfakh 1996; Hsieh et al. 1998; Markham et al. 1999). Plants use a dual antimicrobial/MDR inhibitor combination to combat their microbial pathogens (Stermitz et al. 2000a). For example, berberine,

a weak antimicrobial, is strongly potentiated by 5′-methoxyhydnocarpin, an MDR inhibitor that has no activity on its own. However, presently described plant MDR inhibitors are limited to Gram-positive bacteria, which may explain why there are so few plant pathogens among this group of microorganisms. A synthetic MDR inhibitor active against RND MDRs of Gram-negative species is being developed by Mpex Pharmaceuticals and has entered clinical trials (Lomovskaya and Bostian 2006). NovoBiotic Pharmaceuticals is taking a different approach, based on the assumption that additional classes of broad-spectrum compounds are harbored by the 99% of unculturable microorganisms. Unculturable microorganisms are grown in their natural environment in a diffusion chamber (Kaeberlein et al. 2002) and are then used as a source of secondary metabolites for drug discovery.

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. However, it is not clear that inhibition of a single persister protein will be sufficient to have a desired effect. As mentioned above, a mutant in an essential PlsB protein causes *E. coli* persisters to drop 1-2 log (Spoering et al. 2006), but whether this will be clinically sufficient is not clear.

The requirements for an anti-persister compound are similar to a broad-spectrum antibiotic: it has to penetrate well into cells of both Gram-positive and Gram-negative species. From the above analysis, we can see that the probability of this happening with traditional discovery approaches is 1 drug or less per 40 years. Unlike a conventional antibiotic, however, anti-persister therapy will face an additional hurdle. FDA only requires testing against rapidly growing bacteria, and market conditions are excellent for a new conventional broad-spectrum antibiotic. Why then commit resources to a considerably more challenging anti-persister dual therapy, even if a suitable target will be identified?

In trying to combat persisters, we may have encountered the ultimate adversary. Indeed, persisters evolved through billions of years to perform a single task: survival. During this time, they have encountered all possible harmful compounds, and the inability of any currently employed antibiotics to eliminate persisters provides a sobering view of the magnitude of the challenge. A general recipe in dealing with persisters is then to devise approaches that are not borrowed from and probably do not exist in nature.

2.1 Pulse-Dosing with a Conventional Antibiotic

A disarmingly simple approach to sterilize an infection was first proposed by Bigger in 1944 (Bigger 1944). The idea is to kill all regular cells with a high dose of an antibiotic, then allow the antibiotic levels to drop, which will cause remaining persisters to wake up and convert into regular cells. If a second antibiotic application is administered just after persisters start to grow, an essentially complete sterilization may be achieved. This approach works very well in a test tube, and a *P. aeruginosa* biofilm can be essentially sterilized with two consecutive applications

of a fluoroquinolone (K. Lewis, unpublished data). Pulse-dosing probably does not exist in nature, leaving persisters unprepared to handle this simple regimen.

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 its maximum, which will discourage resistance development. Most importantly, an optimal pulse-dosing regimen will probably vary from patient to patient. However, it seems that patients may have taken solving the problem of intractable persistent infections into their own hands. Individuals who suffer from persistent infections that require a lengthy therapy often do get cured, but why a year-long regimen is better than a week-long one is unclear. An accidental perfect oscillating dosing may very well be responsible for persister eradication in such cases. The patients probably experiment with the dosing by being absent-minded, which sooner or later produces the perfect administration regimen. Curing of persistent infections may therefore result from patient noncompliance. Analyzing how persistent infections are cured may shed some useful light on the likelihood of developing a rational regimen for sterilizing pulse-dosing.

2.1.1 Sterile-Surface Materials

Antiseptics can kill persister cells, but they are obviously toxic and largely unsuitable for systemic applications. Polymeric materials impregnated with antimicrobials have been developed and introduced both as consumer goods and to prevent biofilm formation on catheters. Leaching of the antimicrobial leads to obvious problems: loss of efficacy over time, toxicity in the case of antiseptics, and creation of excellent conditions for developing resistance to a slowly releasing antibiotic.

The ideal approach would be to create a permanently sterile material by covalently attaching an antimicrobial compound to the surface. There is an obvious problem with this approach: once attached to the surface, an antimicrobial molecule loses its mobility and is unable to attack the pathogen. We reasoned that this problem may be solved by linking the antimicrobial to a long, flexible polymeric chain anchored covalently to the surface of a material (Tiller et al. 2001). Quaternary ammonium compounds (QACs) seemed a good choice for the antimicrobial moiety because their target is primarily the microbial membrane (Denyer and Hugo 1991) and they accumulate in the cell driven by the membrane potential (Severina et al. 2001). Attaching a long chain of poly(4-vinyl-*N*-alkylpyridinium bromide) to an amino glass slide produced a material that remained largely sterile (Tiller et al. 2001). Conceptually similar immobilized polymers poly [2-(dimethylamino)ethyl methacrylate] (Lee et al. 2004) and *N*-alkyl-polyethylenimine rapidly depolarized and killed *S. aureus* or *E. coli* cells coming in contact with the surface, with no indication of surviving persisters (Milovic et al. 2005). Importantly, in order to be effective, the sterile-surface polymers have to be long enough to penetrate across the cell envelope (Morgan et al. 2000; Lin et al. 2003), while their shorter versions were ineffective (Lin et al. 2003).

The only known mechanism of resistance to hydrophobic cations is efflux by MDRs (Hsieh et al. 1998). The pathogens are forced to actively accumulate these compounds by electrophoresis across the charged membrane, making these substances especially dangerous (Severina et al. 2001). MDRs probably evolved to counter this threat, and then broadened their spectrum to include other amphipathic compounds as well (Lewis 2001a). However, MDRs evolved to extrude small molecules, not large polymers. Indeed, the activity of a surface modified with *N*-hexyl-PVP was similar against a panel of *S. aureus* strains that consisted of a mutant deleted in the major NorA MDR, a wild type, and a wild-type strain carrying additionally a QacA pump on a natural transmissible plasmid (Lin et al. 2002). Similarly, the soluble analog poly(vinyl-*N*-methylpyridinium iodide) showed the same minimal inhibitory concentrations with all three strains (hexyl-PVP itself is insoluble in water), while the soluble monomer *N*-hexylpyridinium expectedly had the highest activity against the knockout mutant and the lowest against the strain expressing both the NorA and the QacA MDRs. No resistant mutants were found after repeated exposure of bacteria to a surface modified with *N*-alkyl-PEI (Milovic et al. 2005).

It is interesting to consider how MDR resistance is countered in nature. Plants produce QAC-type berberine alkaloids that are actively accumulated by bacteria (Severina et al. 2001), can damage the membrane, and intercalate into the DNA (Jennings and Ridler 1983). From this perspective, berberine appears to be a perfect anti-infective: neither of its targets can be mutated. The only way for bacteria to resist such a compound is by pumping it out via an MDR (Hsieh et al. 1998). Plants, however, in addition to berberine, also make methoxyhydnocarpin, an MDR inhibitor that disables the resistance mechanism of Gram-positive pathogens and acts in synergy with the antimicrobial (Stermitz et al. 2000a). Bacteria, especially Gram-negative species, probably responded by evolving a vast diversity of MDRs, and it is likely that the current state of affairs in this competition is a stalemate between plants producing antimicrobials/MDR inhibitors (Guz et al. 2001; Stermitz et al. 2002; Tegos et al. 2002; Morel et al. 2003; Stermitz et al. 2003; Belofsky et al. 2004, 2006) and bacteria with their large arsenal of MDR pumps. But unlike natural antimicrobials, macromolecular polymers made of amphipathic cations probably do not exist in nature, which would explain why pathogens lack protection from sterile surface materials (Lewis and Klibanov 2005). The attractive properties of cationic polymers are likely to lead to the development of commercial products with sterile surfaces. These materials, however, do not address the need for systemic sterilizing antibiotics.

2.2 Sterilizing Antibiotics

Unlike antiseptics, known target-specific antibiotics do not sterilize an infection. Indeed, persister tolerance is aimed at preventing death from an otherwise bactericidal antibiotic that can only corrupt active targets. Antiseptics that can kill persisters

do not rely on specific targets, damage the cell membrane or DNA/proteins in general, and are obviously toxic. Given this set of facts, developing a single-molecule sterilizing antibiotic does not appear feasible. However, let us consider a perfect antibiotic from first principles. This compound is benign, but a bacterial enzyme converts it into a reactive antiseptic in the cytoplasm. The active molecule does not leave the cytoplasm (because of increased polarity) and attaches covalently to many targets, killing the cell. Irreversible binding to the targets creates a sink that will allow the compound to avoid MDR efflux.

Several existing antimicrobials closely match the properties of this idealized prodrug antibiotic. These are isoniazid, pyrazinamide, ethionamide, and metronidazole. The first three are anti-Mtb drugs, while metronidazole is a broad-spectrum compound acting against anaerobic bacteria. All four compounds convert into active antiseptic-type molecules inside the cell that bind covalently to their targets. It seems to be no accident that prodrug antibiotics make up the core of anti-Mtb drug arsenal. As we have mentioned, *M. tuberculosis* probably forms the most intransigent persisters, and excellent bactericidal properties are a critical feature for any antituberculosis antibiotic. Targets have been identified for isoniazid and ethionamide (Vilcheze et al. 2005), suggesting a relatively limited reactivity for these compounds. The targets are most likely relatively preferred molecules, the top ones on a list that may include many if not most bacterial proteins and DNA. At the same time, the existence of preferred targets indicates that the prodrug products are not that reactive, and there is considerable room for developing better sterilizing antibiotics based on the same principle.

3 Conclusions

Over half a century has passed since the discovery of drug tolerant persisters, but their study is still an emerging field. The presence of persisters in biofilms provides an important incentive to understand their nature. Recent advances in isolating persisters, determining their transcriptome, and finding candidate persister genes are hopeful indications that the pace of progress in understanding this elusive problem is picking up. Formidable obstacles remain, due to difficulty in isolating sufficient amounts of persister cells, the apparent redundancy of persister genes, and the temporary phenotype of these cells. The mechanism of drug tolerance appears to be mechanistically distinct from resistance and is based on shutting down antibiotic targets. Persisters are specialized cells that have evolved to survive all possible natural threats and in confronting persisters, we may have met our ultimate adversary. The challenge is to develop approaches they were unlikely to encounter in nature, such as combination therapies, prodrugs which are activated inside the bacterial cell, sterile-surface materials, and pulse-dosing.

Acknowledgements Work described in this chapter was supported by NIH grant GM061162.

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