Cell Host & Microbe
Review

Microbial Persistence and the Road to Drug Resistance

Nadia R. Cohen,[1,2,3,4,7] Michael A. Lobritz,[1,2,3,4,5,7] and James J. Collins[1,2,3,4,6,*]
[1]Howard Hughes Medical Institute
[2]Department of Biomedical Engineering
[3]Center of Synthetic Biology
Boston University, Boston, MA 02215, USA
[4]Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston, MA 02115, USA
[5]Division of Infectious Diseases, Massachusetts General Hospital, Boston, MA 02114, USA
[6]Boston University School of Medicine, Boston, MA 02118, USA
[7]These authors contributed equally to this work
*Correspondence: jcollins@bu.edu
http://dx.doi.org/10.1016/j.chom.2013.05.009

Microbial drug persistence is a widespread phenomenon in which a subpopulation of microorganisms is able to survive antimicrobial treatment without acquiring resistance-conferring genetic changes. Microbial persisters can cause recurrent or intractable infections, and, like resistant mutants, they carry an increasing clinical burden. In contrast to heritable drug resistance, however, the biology of persistence is only beginning to be unraveled. Persisters have traditionally been thought of as metabolically dormant, nondividing cells. As discussed in this review, increasing evidence suggests that persistence is in fact an actively maintained state, triggered and enabled by a network of intracellular stress responses that can accelerate processes of adaptive evolution. Beyond shedding light on the basis of persistence, these findings raise the possibility that persisters behave as an evolutionary reservoir from which resistant organisms can emerge. As persistence and its consequences come into clearer focus, so too does the need for clinically useful persister-eradication strategies.

Introduction

It was not long after the introduction of antibiotics that the emergence of resistant organisms was first reported (Abraham and Chain, 1940). Around the same time, a similar phenomenon began to frustrate physicians. "Penicillin has undoubtedly saved lives and limbs of patients suffering from staphylococcal infections, but it has not usually cured the disease," wrote Joseph Bigger, an army physician who noticed that antibiotic treatment often failed to completely sterilize soldiers' wounds, resulting in recurrent infections after therapy (Bigger, 1944).

This phenomenon, in which a small subpopulation of microbes survives the lethal effects of a drug, is referred to as "persistence." Persistence is distinct from resistance in that, unlike resistant mutants, persister populations do not expand in the presence of the toxic compound, and population growth resumes only once the drug has been removed. Furthermore, upon retreatment, the recrudescent organisms are as drug sensitive as the initial population, suggesting that unlike resistance, persistence is a nonheritable phenotype (Figure 1).

Many human pathogens cause recurrent infections despite appropriate therapy and in the absence of apparent genetic resistance and thus can be considered persistent. Despite their clinical importance, the protracted nature of these infections as well as the lack of suitable animal models has hampered the study of this phenomenon. Nevertheless, models of persistence in vitro, particularly the study of noninherited drug tolerance in bacterial cultures, biofilms, and microfluidic devices, has helped shed some light on the biological basis of the persister phenotype.

In this review, we discuss the increasing clinical burden of microbial persistence and summarize the current understanding of the mechanisms underlying this phenomenon. In particular, we outline the emerging role of stress responses in potentiating persister survival and highlight the notion that persistence is an actively maintained state rather than solely a consequence of passive growth-rate reduction. Importantly, the stress processes enabling persistence are also implicated in the accelerated acquisition of heritable resistance. Thus, we propose that persisters may serve as an evolutionary reservoir from which resistance might emerge and review the clinical evidence lending credence to this idea. Finally, we discuss potential therapeutic strategies for the prevention or treatment of persistent infections.

Part I: Drug Persistence in Human Disease

Persistence is widespread and has been described in bacteria, fungi, parasites, and even cancerous human cell populations (Table 1). Persistence contributes to the pathogenesis of several notable human infections that require protracted treatment and that relapse after therapy. Furthermore, advances in medical technology allow patients to survive longer with reduced host defenses or in otherwise compromised states. In the absence of appropriate host immunity, pathogens that survive drug treatment are more likely to thrive. As a consequence, the prevalence of persistent infections for which antimicrobial therapy fails to provide a cure is rising.

Persistence in Bacterial Infections

Among of the best-characterized persistent pathogens are *Mycobacteria*, slow-growing aerobic bacteria that include

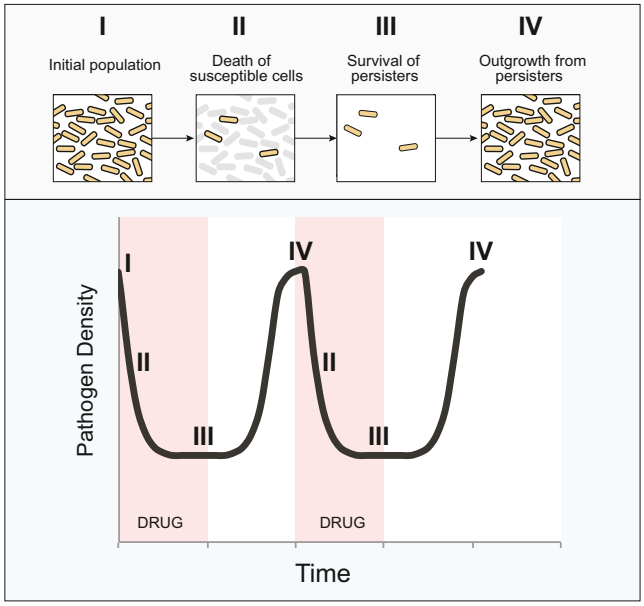

Figure 1. Drug Persistence and Recurrent Infection
Schematic model of killing and persistence kinetics during antimicrobial therapy. Treatment of an initial population of pathogens (I) causes killing of the majority of cells (II) but fails to eradicate a small subset of persisters (III). When antibiotic pressure is removed, persisters resume growth, resulting in recurrent infection of the host (IV). Retreatment results in similar killing kinetics.

Mycobacterium tuberculosis (Mtb) and nontuberculous *Mycobacteria* (NTM). Infections with these organisms require treatment with unusually protracted courses of combinations of antibiotics. Mtb therapy can last up to 12 months, and NTM, such as the *Mycobacterium avium* complex, can require up to 2 years of treatment. Furthermore, despite apparent microbiologic cure, relapse rates of mycobacterial infections are high, underscoring the clinical importance of persistent subpopulations that are undetectable with current diagnostic technology (McCune et al., 1956). Consistent with these observations, detailed studies of infection in mice revealed that treatment of Mtb infections reduces organism titers but fails to sterilize the animal (McCune and Tompsett, 1956).

A variety of other bacteria cause persistent infections in humans. Recurrence of *Staphylococcus aureus* skin, soft tissue, and bloodstream infections, for instance, is common, despite protracted treatment courses (Chong et al., 2013). *S. aureus* is also the frequent cause of infections of foreign devices implanted into humans, including catheters and prosthetic joints. Despite appropriate therapy, infections on these devices recur due to the presence of persistent biofilms. Recurrent infection is also common in the lungs of patients with cystic fibrosis (CF). Though CF-affected lungs are colonized by hundreds of bacterial species, *Pseudomonas aeruginosa* is the most common cause of recurrent pneumonia in these patients (Zhao et al., 2012). Antibiotic treatment of pneumonia in CF-affected patients is the mainstay of therapy; however, antibiotics rarely, if ever, successfully sterilize the airway. In fact, recent evidence indicates that antibiotics have minimal impact on the microbial community structure in these patients, suggesting the existence of antimicrobial tolerance within this bacterial population (Fodor et al., 2012). A relative of *Pseudomonas*, *Burkholderia pseudomallei*, causes meliodosis, a severe systemic infection that can recur months to years after apparent clearance by appropriate antibiotic treatment. Whole-genome sequencing of recrudescent isolates demonstrated nearly clonal organisms, indicating that antimicrobial therapy failed to eradicate the initial infecting population (Hayden et al., 2012).

Persistence in Other Pathogens

Persistence can also complicate therapy for nonbacterial infections. Biofilm-associated infections with fungi, for example, are highly recalcitrant to routine antifungal therapy. Even in the absence of genetic resistance, management of *Candida albicans* infection can frequently require prolonged courses of drugs or the removal of an infected implanted device.

A form of drug tolerance analogous to persistence, referred to as dormancy, has been described for the blood stage of *Plasmodium* species, the protozoal parasites that cause malaria. In dormancy, much like in persistence, a small subpopulation of parasites survives drug treatment and can cause recurrent infection after cessation of therapy. This phenomenon has been described for a wide variety of antimalarial drugs, including mefloquine, atovaquone, and most recently artemisinins, the most commonly used therapy for malaria worldwide (LaCrue et al., 2011; Nakazawa et al., 1995, 2002; Teuscher et al., 2010; Thapar et al., 2005; Witkowski et al., 2010). Similarly, failure of antiprotozoal drugs to clear bloodstream parasites has also been described for *Babesia*, an emerging tick-borne infection that causes a malaria-like illness (Krause et al., 2008). Importantly, as has been observed for other classes of persisters, the bulk of recrudescent parasites appear to remain susceptible to the initial drugs used in treatment.

The Impact of Persistence in Compromised Host States

Although drug persistence is common in microbes and can interfere with treatment, in most cases, antimicrobial therapy succeeds. It is generally accepted that if drugs successfully kill the overwhelming majority of an infecting population, intrinsic host defenses "mop up" the remainder. Advances in medical technology, however, have resulted in the increased prevalence of compromised host states. In these individuals, persisters that would normally be cleared may linger, causing recurrent or intractable infections.

In addition to increased susceptibility to infection, a number of immunocompromised states are also associated with an increased incidence of persistent and recurrent infections. Infection with HIV and its concomitant T cell immunodeficiency state is associated with increased severity and recrudescence of tuberculosis in coinfected individuals (Perriëns et al., 1995). In solid-organ or hematopoetic stem cell transplant recipients, who are chronically immunosuppressed to prevent transplant rejection, infectious complications are a major cause of mortality. Patients with cancer exhibit impaired mucosal barriers, leading to the development of oral candidiasis, which can be kept at bay but not eradicated using local antifungal therapy. Evaluation of isolates of *C. albicans* and *C. glabrata* from such patients demonstrated the presence of drug-tolerant populations and revealed that patients with prolonged fungal carriage had significantly higher levels of persisters (Lafleur et al., 2010). Similarly, infection with the protozoan parasite *Babesia microti*, which responds well to therapy in normal hosts, recrudesces in

Table 1. Human Pathogens Associated with Persistent Infection

Pathogen	Duration of Treatment[a]	Number of Drugs[b]	Persistence Characteristics	Hosts Susceptible to Persistent Infection
Bacteria				
Mycobacteria				
Mycobacterium tuberculosis	months	4	slow clearance	all, particularly HIV
Nontuberculous *Mycobacteria*	months–years	3–4	slow clearance, recurrence	all, particularly bronchiectasis and HIV
Other Bacteria				
Staphylococcus aureus	weeks–lifelong	1–2	biofilm, recurrence	implanted material
Escherichia coli	days–weeks	1	biofilm	urinary catheters
Pseudomonas aeruginosa	days–weeks	1–2	recurrence	CF, immunocompromised
Burkholderia pseudomallei	weeks	1–2	recurrence	all
Burkholderia cenocepacia	days–weeks	1–2	recurrence	CF
Fungi				
Candida species	days–lifelong	1	slow clearance, recurrence	cancer, immunocompromised, HIV
Parasites				
Plasmodium falciparum	days	1–2	recrudescence (artemisinins)	all
Babesia species	weeks–months	1–2	recurrence	immunocompromised
Mammalian Cells				
Tumor cells	months	varies	recurrence	all

CF, cystic fibrosis.
[a]The range of treatment duration.
[b]The typical number of drugs required to achieve apparent eradication of the organism.

immunocompromised individuals despite protracted courses of seemingly appropriate therapy (Krause et al., 2008).

In addition to these impairments in host immunity, compromised host states related to the implantation of cardiac devices, prosthetic joints, devices interacting with the CNS, and other foreign bodies are increasing as the population ages. Treatment of microbial biofilms that form and infect these devices is ineffective and often requires dangerous and costly removal of the implant because of the persistence of a small nucleus of surviving microbes.

Human Cancer Persisters

Noninherited drug tolerance may extend beyond the realm of infectious organisms. A recent study of non-small cell lung tumors identified a subpopulation of reversibly drug-tolerant cancer cells that survived therapy with tyrosine kinase inhibitors (Sharma et al., 2010). Interestingly, this tumor cell line was able to maintain growth in the presence of continued chemotherapy. Furthermore, the drug-tolerant phenotype was mediated through a signal-transduction cascade, resulting in epigenetic modifications within the cell, rather than genetic mutations. Inhibition of histone deacetylase prevented the tolerant state, and it was suggested that the epigenetic modifications involved occurred as part of a cellular stress response program.

These observations underscore the clinical importance of persistence in human disease and indicate that persister formation is a core survival strategy in both prokaryotic and eukaryotic organisms.

Part II: Mechanisms of Drug Persistence

The rising clinical burden of persistence stresses the need to better understand the mechanisms underlying persister formation and maintenance. Despite the paucity of appropriate animal models, the study of persistence in cultures and biofilms, cell-fate mapping experiments using microfluidic devices, and a deepened understanding of antimicrobial toxicity pathways have helped shed light on the basis of persistence in pathogens and model organisms. Bacterial persistence can arise spontaneously or be environmentally induced. These distinct persister classes, as well as the mechanisms thought to allow their generation and survival, are discussed below.

Spontaneous Persistence

The existence of a small fraction of persisters among growing, isogenic microbes has been hypothesized to reflect a population-level strategy of survival in a rapidly changing environment. In this "bet-hedging" view, phenotypic switching of a few organisms to a dormant or protected state occurs spontaneously and continuously in any growing microbial population regardless of the presence of a drug (Kussell et al., 2005).

It has been hypothesized that stochastic variation at the level of gene expression may engender such pre-exisiting phenotypic heterogeneity (Balaban et al., 2004). In particular, toxin/antitoxin (TA) systems, two-gene operons that encode a cellular poison and its antidote and are common throughout the bacterial kingdom, have been implicated in the control of persistence. When in excess, the toxin components typically increase the frequency of bacterial persisters in culture. For instance, in *Escherichia coli*, overexpression of the toxin HipA, or a mutation in this gene that desensitizes HipA to its antitoxin HipB, leads to a 10- to 10,000-fold increase in the frequency of persistence to ampicillin and ciprofloxacin (Falla and Chopra, 1998; Moyed and Bertrand, 1983). Similarly, overexpression of the toxin components of the RelE/B, MazF/E, YafQ/DinJ, MqsR/A, HigB/A, and CcdA/B TA systems enhances persistence to several antibiotics (Maisonneuve et al., 2011; Tripathi

et al., 2012). Whereas ablation of any single toxin gene typically does not dramatically affect persister formation, deletion of multiple TA loci does cause an appreciable drop in the number of persisters (Maisonneuve et al., 2011), suggesting that, although redundant, TA systems play a role in spontaneous persistence.

Variation in the expression of a large number of other genes can also affect persistence or the ability of bacteria to grow in the presence of drug. These include global regulators, genes involved in metabolism, and stress-response components (De Groote et al., 2009; Dhar and McKinney, 2010; Dörr et al., 2009; Hansen et al., 2008; Murakami et al., 2005; Spoering et al., 2006; Wakamoto et al., 2013). In a recent example, *Mycobacterial* growth in the presence of the antibiotic isoniazid was directly correlated to stochastic variation in the pulsatile expression of KatG, a catalase peroxidase required for processing and activation of the drug. Specifically, slow-pulsing cells, which processed less drug, survived longer than fast-pulsing bacteria (Wakamoto et al., 2013).

Genes affecting survival under stress may be particularly prone to epigenetic plasticity. Although this question has not been widely addressed, large-scale transcriptional analysis of a clonal population of *Plasmodium falciparum* found that stochasticity is an intrinsic property of the expression of gene families involved in host-parasite interactions, suggesting that variability in these genes might improve the persistence of an infecting population (Rovira-Graells et al., 2012). In addition to stochastic gene-expression variation, phenotypic heterogeneity in drug sensitivity may result from built-in deterministic mechanisms. For instance, asymmetric growth of *Mycobacterium smegmatis* gives rise to daughter cells of different sizes and with different elongation rates, and the slower-growing progeny are less susceptible to multiple drugs (Aldridge et al., 2012).

Environmentally Induced Persistence

A number of environmental signals can modulate the level of persistence in an isogenic microbial population. This phenomenon has been best studied in bacterial cultures, wherein a variety of noxious cues have been found to increase the frequency of persistence (Figure 2).

Heat shock, for example, increases the survival of both *Acinetobacter baumannii* and *P. aeruginosa* in the presence of aminoglycosides or β-lactams, respectively (Cardoso et al., 2010; Murakami et al., 2005). Oxidative stress and DNA damage caused by treatment with paraquat, hydrogen peroxide, or sublethal doses of antibiotics promote persistence to fluoroquinolone antibiotics in both *E. coli* and *P. aeruginosa* (Dörr et al., 2009; Möker et al., 2010; Vega et al., 2012; Wu et al., 2012). Similarly, bacterial envelope stress has been associated with enhanced persister formation (Murakami et al., 2005; Poole, 2012), as has signaling mediated by soluble quorum sensing (QS) molecules including phenazine pyocyanin in *P.aeruginosa*, peptide alarmones in *Streptococcus mutans*, and indole, a stationary-phase QS molecule secreted and sensed by a wide range of bacteria (Kayama et al., 2009; Leung and Lévesque, 2012; Möker et al., 2010; Vega et al., 2012). Nutrient starvation and diauxic carbon-source transitions can also induce persistence (Amato et al., 2013; Betts et al., 2002; Fung et al., 2010; Nguyen et al., 2011). In fungi, environmental conditions have similarly been suggested to affect drug susceptibility (Kucharíková et al., 2011; Pettit et al., 2010). Biofilm-encased or stationary-phase cells, which are deprived of certain nutrients and may be subjected to QS signaling, exhibit persistence at levels typically orders of magnitude higher than those of logarithmically growing organisms (Keren et al., 2004; LaFleur et al., 2006).

Importantly, data suggest that in some cases, bacterial persistence that appears spontaneous may in fact have been environmentally induced. Specifically, maintenance of *E. coli* cultures in log phase through repeated reinoculation leads to the loss of a detectable persister population. These data suggest that persisters found in actively growing, low-stress populations may in fact represent leftover cells from high-stress stationary-phase inoculums rather than actively growing bacteria that have switched phenotypes spontaneously (Keren et al., 2004).

Together, these data underscore the role of environmental parameters such as nutrient availability, population density, and oxidative stress in the modulation of the persistence levels in a population.

Stress-Response Pathways Enable Drug Persistence

Whether persistence is acquired spontaneously or is environmentally induced, there is increasing evidence, particularly in bacteria, for the active involvement of various and interconnected intracellular stress responses (Poole, 2012).

In bacteria, both spontaneous and stress-induced persistence have been shown to depend on the stringent response (SR). The SR, which can be triggered by a range of cues, depends on RelA- and SpoT-mediated synthesis of (p)ppGpp, an alarmone that broadly modulates gene expression to promote cell survival (Potrykus and Cashel, 2008). In *E. coli hipA7* mutants, which express a gain-of-function mutation in the HipA toxin and exhibit elevated spontaneous persister frequencies (Balaban et al., 2004), simultenously deleting *relA* and *spoT* eliminates persistence (Korch et al., 2003). The broad toxic effects of HipA on nucleic acid and protein synthesis (Korch and Hill, 2006) may be responsible for triggering the SR. In addition, genetic knockout of *relA* and *spoT* in both *P. aeruginosa* and *E. coli* diminishes starvation-induced persistence, biofilm drug tolerance, and persistence in vivo (Fung et al., 2010; Nguyen et al., 2011). Recently, *E. coli* persistence induced by diauxic carbon-source transitioning has also been found to require the SR (Amato et al., 2013). Starvation-induced drug tolerance in *M. tuberculosis* has been suggested to depend on Rel_{Mtb}, a RelA homolog (Betts et al., 2002). Interestingly, a gain-of-function mutation in *relA* causing permanent activation of the SR generated a high-persistence phenotype in a primary isolate of *S. aureus* identified in human infection (Gao et al., 2010).

Activation of the bacterial SOS response to DNA damage, much like the SR, has been associated with induction or maintenance of persistence. *E. coli* mutants lacking *recA*, *recB*, or *lexA*, key SOS response genes, are more susceptible to quinolones, and in the presence of these drugs, the frequency of both spontaneous and induced persistence is reduced by 10- to 100-fold (Dörr et al., 2009; Fung et al., 2010; Wu et al., 2012). Conversely, constitutive expression of SOS response genes enhances drug tolerance (Dörr et al., 2009). Thus, persistence to quinolones is aided and partially depends on the cell's ability to repair DNA damage.

A number of other stress-response pathways have been implicated in persister formation. These include the oxidative stress

response, which depends on OxyR and SoxS, and the phage-shock response, both of which are involved in indole-induced persistence in *E. coli* (Grant and Hung, 2013; Vega et al., 2012; Wu et al., 2012). Genes regulating the heat-shock response have been reported to play a role in tolerance to aminoglycosides (Cardoso et al., 2010), and the alternative sigma factor RpoS, which coordinates the general stress response, is important in QS-induced persistence in *P. aeruginosa* (Kayama et al., 2009; Murakami et al., 2005).

Taken together, these data suggest that centralized stress responses are at the core of the persister phenotype. Both environmental factors and stochastic endogenous processes can give rise to stress and thus contribute to the generation of multi-drug-tolerant persisters.

Mechanisms of Survival

Most stress responses lead directly or indirectly to a slowing or stalling of bacterial growth and division. This slowed or arrested cellular growth has been suggested as the major factor underlying drug persistence, given that the ability of antibiotics to kill bacteria is generally proportional to their growth rate (Eng et al., 1991). Reduced growth rates have indeed been correlated with increased drug persistence both in vitro (Aldridge et al., 2012; Balaban et al., 2004; Keren et al., 2004; Shah et al., 2006) and in vivo, when bacterial growth tapers over the course of an infection due to immune pressure or lack of nutrients (Levin and Rozen, 2006). The reduced rates of DNA replication, translation, cell-wall synthesis, and metabolism directly targeted by antibiotics have been assumed to account for the relative drug tolerance of dormant bacteria. Although stalled biosynthesis probably promotes persistence, its effects are difficult to untangle from those of accompanying stress-response processes, and it is becoming clear that many active cellular processes occurring in parallel with growth-rate reduction are central for cellular survival in a toxic environment.

For instance, a number of active intracellular detoxification mechanisms that can be triggered by stress play an important role in persistence. Multidrug efflux pumps are upregulated in response to various cues, including oxidative stress and QS (Hirakawa et al., 2005), and can contribute to both in vitro and in vivo persistence. For example, paraquat-induced persistence in *E. coli* in vitro is dependent on the AcrAB-TolC multidrug efflux system (Wu et al., 2012). Similarly, drug tolerance in a *Mycobacterial* infection model was reported to depend on drug efflux pumps induced by macrophage-mediated oxidative stress (Adams et al., 2011). In some cases, persisters exhibit a reversible defect in drug uptake (Allison et al., 2011b). Beyond active efflux, detoxification can take other forms. For example, the exposure of bacteria to antibiotics can enhance their synthesis of nitric oxide, which chemically modifies certain drugs and inactivates them (Gusarov et al., 2009; Shatalin et al., 2011). In *Mycobacteria*, stress can also promote alternative metabolic pathways that produce lower levels of reactive oxygen species (ROS), thus elevating the threshold for antibiotic-mediated death (Baek et al., 2011). Finally, generation of ROS following antibiotic exposure has been proposed to contribute to the lethal effects of antimicrobials (Dwyer et al., 2007; Foti et al., 2012; Kohanski et al., 2007, 2008; Wang and Zhao, 2009). Although the role of ROS in antibiotic-mediated bacterial killing has recently become a matter of debate (Keren et al., 2013; Liu and Imlay, 2013), active mechanisms of oxidative stress relief have been found to promote drug tolerance. For example, activation of catalase and superoxide dismutase, antioxidant enzymes, promotes stress-induced drug persistence and biofilm-associated persistence in bacteria and fungi (Belenky and Collins, 2011; Nguyen et al., 2011; Shatalin et al., 2011; Van Acker et al., 2013).

In addition to detoxification, stress can promote active microbial mechanisms of physical sequestration. For instance, bacterial surface adhesiveness and the subsequent formation of protective biofilms can be enhanced in response to sublethal antibiotic treatment and other stresses (He et al., 2012; Hoffman et al., 2005). Although tolerance mechanisms in biofilms are still a subject of intense investigation, it is clear that mechanisms other than dormancy are at play (Høiby et al., 2010).

Interestingly, persistence and cellular growth may not always be incompatible. Although spontaneous *E. coli* persisters have been shown to be growth arrested (Balaban et al., 2004), a microfluidics-based study following the fate of individual *Mycobacteria* during antibiotic treatment found that the apparent stability of persister numbers was in fact due to a dynamic state of balanced death and division, rather than generally arrested growth (Wakamoto et al., 2013).

Together, these recent studies challenge the prevailing view that persistence is solely linked to passive dormancy and suggest that these cells are engaged in a host of active processes that allow survival (Adams et al., 2011; Nguyen et al., 2011; Wakamoto et al., 2013).

Part III: Persistence-Enabling Stress Responses Accelerate Acquisition of Resistance

The discovery that cellular activity and division can occur during persistence raises the possibility that persisters function as an intermediate state in the elaboration of heritable drug resistance. Residual cell division combined with active survival mechanisms could support or even enhance the development of heritable genetic changes, because many of the same stress-response programs important for the generation and survival of persisters can also accelerate genome-wide mutagenesis and horizontal gene transfer. Thus, persisters may represent a pool of adaptively evolving organisms from which resistant mutants can emerge (Figure 2).

Stress Responses Promote Adaptive Mutation

Although in theory low rates of cell division in persisters under drug pressure may suffice to give rise to resistance-conferring mutations, stress responses might vastly accelerate this process. Recently, exogenous stress has been shown to promote genome instability, or "adaptive mutation," leading to fast-tracked population diversification.

Recent systems-level analyses have uncovered that overlapping stress responses (SR and SOS) function as upstream hubs of a program of adaptive mutagenesis in *E. coli*, *S. aureus*, and *P. aeruginosa* (Al Mamun et al., 2012; Cirz et al., 2006, 2007). For example, activation of the SR by starvation stress increases basal mutation rates in *E. coli* through activation of the error-prone DinB polymerase (Petrosino et al., 2009). Similarly, mutagenic polymerases are induced in *S. aureus* and *P. aeruginosa* following induction of the SOS response (Cirz et al., 2006, 2007).

In addition to the activity of microbial stress-response systems, bactericidal antibiotic therapy alone has been shown to

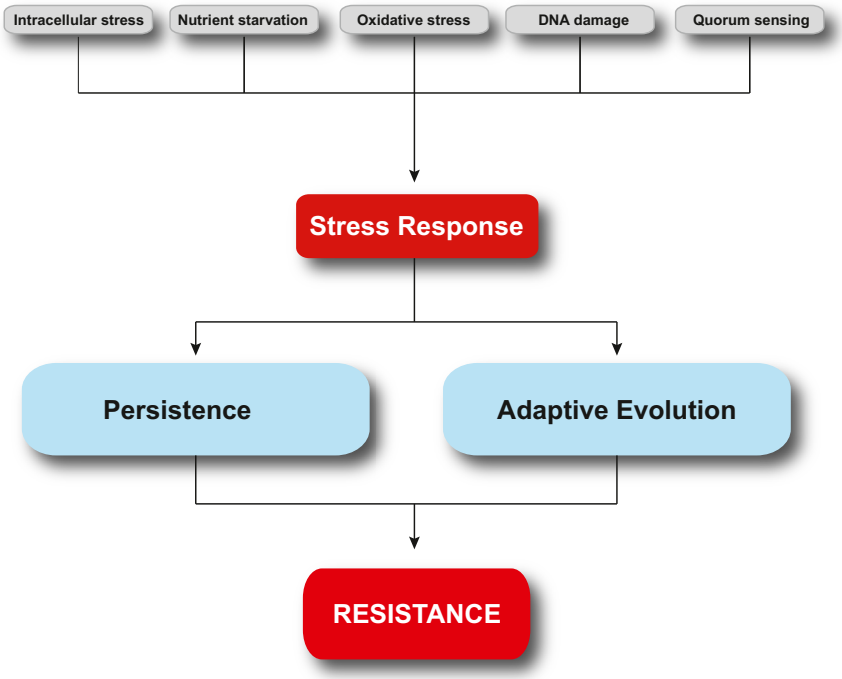

Figure 2. Stress Responses Link Persistence, Adaptive Evolution, and Resistance

In this model, microbial responses to endogenous or exogenous stresses promote survival as well as genetic plasticity. Persistent organisms undergo rapid adaptive evolution and can function as a reservoir for the elaboration of drug resistance.

increase rates of mutagenesis. Treatment of bacteria with sublethal doses of bactericidal antibiotics results in genome-wide mutations through stimulation of ROS production and through RpoS-mediated activation of the error-prone polymerase PolIV (Gutierrez et al., 2013; Kohanski et al., 2010; Nair et al., 2013). Selection of such resistance mutations by antibiotics can occur at drug concentrations orders of magnitude below the inhibitory level of susceptible bacteria (Gullberg et al., 2011).

Interestingly, a link between ROS-specific mutagenesis by sublethal antibiotics and microbial stress-response pathways has recently been demonstrated. In *E. coli* lacking the SR (both *relA* and *spoT*), treatment of cultures with sublethal antibiotics failed to generate adaptive resistance, a phenomenon that was linked to reduced rates of oxidative stress in the SR null organism (Nguyen et al., 2011). This suggests that an intact SR pathway is required for an antibiotic-stress-caused adaptive mutation to develop.

The structure of bacterial communities may also play an important role in the occurrence of adaptive mutations (Zhang et al., 2011). Higher mutation rates have been found in biofilm-associated *P. aeruginosa* (Driffield et al., 2008) and *S. aureus* (Ryder et al., 2012) than in planktonic cultures, and these increased rates appear to be linked to higher levels of oxidative stress in the biofilm community. Thus, features consistent with persister formation—exposure to bactericidal antibiotics, activation of stress-response pathways, and biofilm association—create a suitable environment for adaptive mutation to occur within this subpopulation.

Stress Responses Promote Horizontal Gene Transfer

A second major mechanism by which antibiotic resistance is elaborated is by the sharing of resistance determinants on mobile genetic elements. Stress-response pathways, particularly the SOS, have also been found to promote horizontal gene transfer in bacteria. Induction of the SOS response by the quinolone class of antibiotics increases horizontal gene transfer frequency in *E. coli* and *Vibrio cholera* (Beaber et al., 2004), specifically promoting the sharing of integrative conjugative elements that result in resistance to aminoglycosides, lincosamides, and antifolate antibiotics.

In organisms that lack a traditional SOS system, alternative stress-response pathways have been found to promote gene transfer. In *Streptococcus pneumoniae*, which lacks an SOS system, competence induction by antibiotics has been linked to activation of QS signals as a response to the management of misfolded proteins caused by aminoglycoside treatment (Prudhomme et al., 2006; Stevens et al., 2011). Higher rates of phage transduction, which can disseminate antibiotic resistance determinants, have also been reported with treatment of some antibiotics (Zhang et al., 2000). Interestingly, environmental stress from sources other than antibiotics has similarly been shown to increase rates of horizontal gene transfer in bacterial communities (Stecher et al., 2012). Biofilm formation, in addition to increasing rates of adaptive mutation, can also promote horizontal gene transfer in *S. aureus* (Savage et al., 2013). Thus, stress-response pathways and microbial community structures that favor the development of persisters can also potentiate horizontal gene transfer.

Part IV: Is Persistence Linked to Drug Resistance In Vivo?

Just as our understanding of persisters in vivo remains limited, characterization of how drug resistance emerges in patients remains largely obscure. The increasing use of whole-genome sequencing of microbes is beginning to shed light on pathogen dynamics during persistent infection.

For example, longitudinal whole-genome sequencing of *S. aureus* isolates in a patient with persistent infection demonstrated the accumulation over several months of a small number of point mutations associated with antibiotic resistance that would not have been detected by standard laboratory practice (Mwangi et al., 2007). This dynamic structure of bacterial populations was further explored using whole-genome sequencing of a CF pneumonia outbreak strain, *Burkholderia dolosa*, in multiple consecutive patients. In this longitudinal analysis, near-continual parallel adaptive evolution occurred among isolates in very similar genetic loci involved in antibiotic resistance and oxygen

tolerance across all patients (Lieberman et al., 2011). Similarly, whole-genome sequencing of recrudescent infection with *Burkholderia pseudomallei* established that infection recurrences did not result from reinfection but rather from regrowth of persisting organisms. The study further identified in these isolates a number of antibiotic-resistance-conferring mutations. Although these antibiotic-resistant mutants may have been present in the initial infecting population and subsequently selected for, the nature of the genetic changes and the detection of specific mutations typically associated with recurrent isolates, but not with environmental strains, suggested that evolution toward resistance had occurred in the host (Hayden et al., 2012).

Whole-genome sequencing of populations of microbes, rather than just individual clones, has also disclosed significantly greater community complexity than previously appreciated. This is particularly important in the context of antibiotic resistance. In vitro, populations of bacteria under antibiotic pressure have been found to be composed of mixed subpopulations of susceptible and resistant organisms. A small number of altruistic, resistant organisms sensing environmental stress can lead to a population-wide drug-tolerant state by secreting indole (Lee et al., 2010). In an in vivo example, whole-genome sequencing of Mtb populations in sputum from patients who experienced recrudescent infection, including analysis of very-low-abundance variants in the population, revealed a remarkable amount of coexisting genetic diversity and the frequent emergence of resistant strains at low frequencies (Sun et al., 2012).

The links between dormancy in *Plasmodium* species and the development of resistance to antimalarial compounds in vivo remain unclear. Resistance to artemisinin in *P. falciparum* has recently emerged and is likely to spread more widely (Miotto et al., 2013). Artemisinin resistance was originally identified by a delay in the clearance of parasites from the bloodstream (Amaratunga et al., 2012; Dondorp et al., 2009). A recent SNP analysis of parasites with delayed clearance identified four associated polymorphisms, including mutations associated with a DNA damage tolerance pathway (Takala-Harrison et al., 2013). Mathematical modeling experiments have suggested that reduced artemesinin susceptibility is related to reduced ring-stage susceptibility, the life stage associated with dormancy (Saralamba et al., 2011). Modeling has also suggested that recrudescence is an important clinical feature for the dissemination of de novo drug resistance (White et al., 2009). Though these associations provoke interest in a potential link between dormancy and artemesinin resistance, more research is needed to draw meaningful conclusions.

Part V: Toward Therapeutic Eradication of Persisters

As discussed above, not only do microbial persisters thwart our efforts to treat infection, but they may also act as reservoirs of actively and adaptively evolving organisms. Although a causal relationship between persistence and resistance remains to be definitively established, the possibility that persisters may give rise to resistant mutants further underscores the importance of developing methods to eradicate such phenotypically drug-refractory subpopulations. As our mechanistic understanding of persistence deepens, new strategies for targeting these cells specifically or for enhancing their susceptibility to killing by existing antimicrobials are being devised (Allison et al., 2011a).

One possibility is targeting the stress responses that coordinate the persistent phenotype and the generation of resistance. Although work in this area is still in its infancy, a few promising studies have begun to explore this approach (Cirz and Romesberg, 2007). For example, inhibition of the SOS response either by deletion of *recA* or by overexpression of an uncleavable mutant of the LexA repressor potentiates killing by quinolones, aminoglycosides, and β-lactams (Kohanski et al., 2007; Lu and Collins, 2009). Supplementing antibiotic therapy with engineered bacteriophage constitutively expressing an uncleavable LexA repressor improved survival in a murine model of acute *E. coli* infection and even improved killing of genetically drug-resistant mutants. Furthermore, combination therapy with these bacteriophages substantially reduced the rate of antibiotic-induced mutation and resistance emergence in sublethally treated bacteria (Lu and Collins, 2009). Small-molecule screens have identified RecA inhibitors that might similarly enhance antibiotic activity and prevent adaptive mutation in bacterial pathogens (Wigle et al., 2009). Similarly, preventing the derepression of the SOS response or ablating SOS-induced alternative polymerases prevents UV-induced adaptive mutation in *S. aureus* (Cirz et al., 2007).

Interestingly, bacteriophage engineered to interfere with the oxidative stress response by overexpressing SoxR also potentiates antibiotic therapy (Lu and Collins, 2009). Other findings suggest that drugs targeting the SR (Wexselblatt et al., 2012) or interfering with envelope-repair pathways (Lee et al., 2009) may also help prevent persister formation or enhance bactericidal antibiotic activity.

Another strategy is interference with persister survival mechanisms. Therapies could target residual metabolic activity in persisting organisms, for example. Indeed, inhibition of the mycobacterial proteasome or ATP synthase has been shown to promote the death of nongrowing *M. tuberculosis* (Andries et al., 2005; Gandotra et al., 2007; Lin et al., 2009). Because of the protective role biofilms play in drug- and immune-related persistence, the processes required for their formation are also being investigated for potential targets. Small-molecule screens have been designed for finding candidate biofilm inhibitors (Cegelski et al., 2009; Junker and Clardy, 2007). Among others, one group discovered compounds that prevent the biogenesis of pili and curli, extracellular bacterial fibers that mediate substrate attachment in the initial phases of biofilm formation (Aberg and Almqvist, 2007). Although treatment susceptibility was not assessed, these compounds effectively reduced the virulence of uropathogenic *E. coli* in a murine infection model (Cegelski et al., 2009). Therapeutic strategies to disaggregate existing biofilms are also being considered (Boles and Horswill, 2008; Lu and Collins, 2007). Detoxification mechanisms such as efflux pumps are being investigated as new antibiotic targets or as adjuvants to existing therapies (Fernández and Hancock, 2012). Persisters can also be antagonized by potentiating antibiotic toxicity though the enhancement of drug uptake. For example, aminoglycoside uptake by persisters and the efficacy of aminoglycoside treatment of catheter-associated *E. coli* infection in mice can be increased through coadministration of specific metabolites that help power the transmembrane proton motive force (Allison et al., 2011b; Lu and Collins, 2009). Finally, the use of adjuvants that maximize antibiotic-induced intracellular ROS

production is also being explored (Brynildsen et al., 2013; Grant et al., 2012).

Drug screens have traditionally focused on identifying compounds lethal to microbes in stress-free in vitro culture environments. Although this has been a successful way to identify drug targets, it is becoming clear that many microbial processes not required for idealized life in vitro are essential for survival over the course of infection and treatment in humans (Barczak and Hung, 2009). Such processes, which include both persistence and virulence, are intimately linked to dynamic and complex networks of stress responses that orchestrate phenotypic adaptation. Learning more about these processes and their role in infection is likely to yield a rich trove of potential new drug targets and hopefully will lead to more effective therapies against persistent, relapsing, and resistant organisms.

Conclusion and Future Directions

Persistent and recurrent infections in humans are common, and their frequency is rising. Despite this, our understanding of antimicrobial treatment failure remains limited. Though insight into microbial drug tolerance has been gained using in vitro systems, the persister phenotype has remained only partially characterized. Going forward, innovative approaches to persister isolation and molecular definition are urgently needed to more deeply explore mechanisms involved in induction, maintenance, and exit from the persister state. Furthermore, animal models that faithfully recapitulate human infection and treatment courses should be devised to corroborate culture-based data. Lastly, for exploring persistence and resistance dynamics in vivo, longitudinal studies of microbial population size, diversity, and evolution occurring within individual patients during the course of infection and treatment should be initiated. Although indirect evidence suggests that the persister state may promote the emergence of resistance, no definitive experiment has established that this is the case. An integrative approach that compares the results of culture systems, animal models, and natural history cohorts of primary human infection will facilitate the elucidation of persistence mechanisms, help to determine the magnitude of the impact of persistence to human treatment failure and drug resistance, and usher in new approaches to the treatment and eradication of infectious diseases.

ACKNOWLEDGMENTS

We thank Drs. Kara Lassen, Ahmad S. Khalil, Peter Belenky, and Rebecca S. Shapiro for critical input and reading of the manuscript. This work was supported by funding from the Wyss Institute Clinical Fellowship program and the Howard Hughes Medical Institute.

REFERENCES

Aberg, V., and Almqvist, F. (2007). Pilicides-small molecules targeting bacterial virulence. Org. Biomol. Chem. *5*, 1827–1834.

Abraham, E.P., and Chain, E. (1940). An Enzyme from Bacteria able to Destroy Penicillin. Nature *146*, 837.

Adams, K.N., Takaki, K., Connolly, L.E., Wiedenhoft, H., Winglee, K., Humbert, O., Edelstein, P.H., Cosma, C.L., and Ramakrishnan, L. (2011). Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. Cell *145*, 39–53.

Al Mamun, A.A., Lombardo, M.J., Shee, C., Lisewski, A.M., Gonzalez, C., Lin, D., Nehring, R.B., Saint-Ruf, C., Gibson, J.L., Frisch, R.L., et al. (2012). Identity and function of a large gene network underlying mutagenic repair of DNA breaks. Science *338*, 1344–1348.

Aldridge, B.B., Fernandez-Suarez, M., Heller, D., Ambravaneswaran, V., Irimia, D., Toner, M., and Fortune, S.M. (2012). Asymmetry and aging of mycobacterial cells lead to variable growth and antibiotic susceptibility. Science *335*, 100–104.

Allison, K.R., Brynildsen, M.P., and Collins, J.J. (2011a). Heterogeneous bacterial persisters and engineering approaches to eliminate them. Curr. Opin. Microbiol. *14*, 593–598.

Allison, K.R., Brynildsen, M.P., and Collins, J.J. (2011b). Metabolite-enabled eradication of bacterial persisters by aminoglycosides. Nature *473*, 216–220.

Amaratunga, C., Sreng, S., Suon, S., Phelps, E.S., Stepniewska, K., Lim, P., Zhou, C., Mao, S., Anderson, J.M., Lindegardh, N., et al. (2012). Artemisinin-resistant Plasmodium falciparum in Pursat province, western Cambodia: a parasite clearance rate study. Lancet Infect. Dis. *12*, 851–858.

Amato, S.M., Orman, M.A., and Brynildsen, M.P. (2013). Metabolic control of persister formation in *Escherichia coli*. Mol. Cell *50*, 475–487.

Andries, K., Verhasselt, P., Guillemont, J., Göhlmann, H.W., Neefs, J.M., Winkler, H., Van Gestel, J., Timmerman, P., Zhu, M., Lee, E., et al. (2005). A diarylquinoline drug active on the ATP synthase of Mycobacterium tuberculosis. Science *307*, 223–227.

Baek, S.H., Li, A.H., and Sassetti, C.M. (2011). Metabolic regulation of mycobacterial growth and antibiotic sensitivity. PLoS Biol. *9*, e1001065.

Balaban, N.Q., Merrin, J., Chait, R., Kowalik, L., and Leibler, S. (2004). Bacterial persistence as a phenotypic switch. Science *305*, 1622–1625.

Barczak, A.K., and Hung, D.T. (2009). Productive steps toward an antimicrobial targeting virulence. Curr. Opin. Microbiol. *12*, 490–496.

Beaber, J.W., Hochhut, B., and Waldor, M.K. (2004). SOS response promotes horizontal dissemination of antibiotic resistance genes. Nature *427*, 72–74.

Belenky, P., and Collins, J.J. (2011). Microbiology. Antioxidant strategies to tolerate antibiotics. Science *334*, 915–916.

Betts, J.C., Lukey, P.T., Robb, L.C., McAdam, R.A., and Duncan, K. (2002). Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling. Mol. Microbiol. *43*, 717–731.

Bigger, J.W. (1944). Treatment of staphylococcal infections with penicillin by intermittent sterilisation. Lancet *244*, 497–500.

Boles, B.R., and Horswill, A.R. (2008). Agr-mediated dispersal of Staphylococcus aureus biofilms. PLoS Pathog. *4*, e1000052.

Brynildsen, M.P., Winkler, J.A., Spina, C.S., MacDonald, I.C., and Collins, J.J. (2013). Potentiating antibacterial activity by predictably enhancing endogenous microbial ROS production. Nat. Biotechnol. *31*, 160–165.

Cardoso, K., Gandra, R.F., Wisniewski, E.S., Osaku, C.A., Kadowaki, M.K., Felipach-Neto, V., Haus, L.F., and Simão, Rde.C. (2010). DnaK and GroEL are induced in response to antibiotic and heat shock in Acinetobacter baumannii. J. Med. Microbiol. *59*, 1061–1068.

Cegelski, L., Pinkner, J.S., Hammer, N.D., Cusumano, C.K., Hung, C.S., Chorell, E., Aberg, V., Walker, J.N., Seed, P.C., Almqvist, F., et al. (2009). Small-molecule inhibitors target Escherichia coli amyloid biogenesis and biofilm formation. Nat. Chem. Biol. *5*, 913–919.

Chong, Y.P., Park, S.J., Kim, H.S., Kim, E.S., Kim, M.N., Park, K.H., Kim, S.H., Lee, S.O., Choi, S.H., Jeong, J.Y., et al. (2013). Persistent Staphylococcus aureus bacteremia: a prospective analysis of risk factors, outcomes, and microbiologic and genotypic characteristics of isolates. Medicine (Baltimore) *92*, 98–108.

Cirz, R.T., and Romesberg, F.E. (2007). Controlling mutation: intervening in evolution as a therapeutic strategy. Crit. Rev. Biochem. Mol. Biol. *42*, 341–354.

Cirz, R.T., O'Neill, B.M., Hammond, J.A., Head, S.R., and Romesberg, F.E. (2006). Defining the Pseudomonas aeruginosa SOS response and its role in the global response to the antibiotic ciprofloxacin. J. Bacteriol. *188*, 7101–7110.

Cirz, R.T., Jones, M.B., Gingles, N.A., Minogue, T.D., Jarrahi, B., Peterson, S.N., and Romesberg, F.E. (2007). Complete and SOS-mediated response of Staphylococcus aureus to the antibiotic ciprofloxacin. J. Bacteriol. *189*, 531–539.

De Groote, V.N., Verstraeten, N., Fauvart, M., Kint, C.I., Verbeeck, A.M., Beullens, S., Cornelis, P., and Michiels, J. (2009). Novel persistence genes in Pseudomonas aeruginosa identified by high-throughput screening. FEMS Microbiol. Lett. *297*, 73–79.

Dhar, N., and McKinney, J.D. (2010). Mycobacterium tuberculosis persistence mutants identified by screening in isoniazid-treated mice. Proc. Natl. Acad. Sci. USA *107*, 12275–12280.

Dondorp, A.M., Nosten, F., Yi, P., Das, D., Phyo, A.P., Tarning, J., Lwin, K.M., Ariey, F., Hanpithakpong, W., Lee, S.J., et al. (2009). Artemisinin resistance in Plasmodium falciparum malaria. N. Engl. J. Med. *361*, 455–467.

Dörr, T., Lewis, K., and Vulić, M. (2009). SOS response induces persistence to fluoroquinolones in Escherichia coli. PLoS Genet. *5*, e1000760.

Driffield, K., Miller, K., Bostock, J.M., O'Neill, A.J., and Chopra, I. (2008). Increased mutability of Pseudomonas aeruginosa in biofilms. J. Antimicrob. Chemother. *61*, 1053–1056.

Dwyer, D.J., Kohanski, M.A., Hayete, B., and Collins, J.J. (2007). Gyrase inhibitors induce an oxidative damage cellular death pathway in Escherichia coli. Mol. Syst. Biol. *3*, 91.

Eng, R.H., Padberg, F.T., Smith, S.M., Tan, E.N., and Cherubin, C.E. (1991). Bactericidal effects of antibiotics on slowly growing and nongrowing bacteria. Antimicrob. Agents Chemother. *35*, 1824–1828.

Falla, T.J., and Chopra, I. (1998). Joint tolerance to beta-lactam and fluoroquinolone antibiotics in Escherichia coli results from overexpression of hipA. Antimicrob. Agents Chemother. *42*, 3282–3284.

Fernández, L., and Hancock, R.E. (2012). Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. Clin. Microbiol. Rev. *25*, 661–681.

Fodor, A.A., Klem, E.R., Gilpin, D.F., Elborn, J.S., Boucher, R.C., Tunney, M.M., and Wolfgang, M.C. (2012). The adult cystic fibrosis airway microbiota is stable over time and infection type, and highly resilient to antibiotic treatment of exacerbations. PLoS ONE *7*, e45001.

Foti, J.J., Devadoss, B., Winkler, J.A., Collins, J.J., and Walker, G.C. (2012). Oxidation of the guanine nucleotide pool underlies cell death by bactericidal antibiotics. Science *336*, 315–319.

Fung, D.K., Chan, E.W., Chin, M.L., and Chan, R.C. (2010). Delineation of a bacterial starvation stress response network which can mediate antibiotic tolerance development. Antimicrob. Agents Chemother. *54*, 1082–1093.

Gandotra, S., Schnappinger, D., Monteleone, M., Hillen, W., and Ehrt, S. (2007). In vivo gene silencing identifies the Mycobacterium tuberculosis proteasome as essential for the bacteria to persist in mice. Nat. Med. *13*, 1515–1520.

Gao, W., Chua, K., Davies, J.K., Newton, H.J., Seemann, T., Harrison, P.F., Holmes, N.E., Rhee, H.W., Hong, J.I., Hartland, E.L., et al. (2010). Two novel point mutations in clinical Staphylococcus aureus reduce linezolid susceptibility and switch on the stringent response to promote persistent infection. PLoS Pathog. *6*, e1000944.

Grant, S.S., and Hung, D.T. (2013). Persistent bacterial infections, antibiotic tolerance, and the oxidative stress response. Virulence *4*, 273–283.

Grant, S.S., Kaufmann, B.B., Chand, N.S., Haseley, N., and Hung, D.T. (2012). Eradication of bacterial persisters with antibiotic-generated hydroxyl radicals. Proc. Natl. Acad. Sci. USA *109*, 12147–12152.

Gullberg, E., Cao, S., Berg, O.G., Ilbäck, C., Sandegren, L., Hughes, D., and Andersson, D.I. (2011). Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog. *7*, e1002158.

Gusarov, I., Shatalin, K., Starodubtseva, M., and Nudler, E. (2009). Endogenous nitric oxide protects bacteria against a wide spectrum of antibiotics. Science *325*, 1380–1384.

Gutierrez, A., Laureti, L., Crussard, S., Abida, H., Rodríguez-Rojas, A., Blázquez, J., Baharoglu, Z., Mazel, D., Darfeuille, F., Vogel, J., and Matic, I. (2013). β-lactam antibiotics promote bacterial mutagenesis via an RpoS-mediated reduction in replication fidelity. Nat. Commun. *4*, 1610.

Hansen, S., Lewis, K., and Vulić, M. (2008). Role of global regulators and nucleotide metabolism in antibiotic tolerance in Escherichia coli. Antimicrob. Agents Chemother. *52*, 2718–2726.

Hayden, H.S., Lim, R., Brittnacher, M.J., Sims, E.H., Ramage, E.R., Fong, C., Wu, Z., Crist, E., Chang, J., Zhou, Y., et al. (2012). Evolution of Burkholderia pseudomallei in recurrent melioidosis. PLoS ONE *7*, e36507.

He, H., Cooper, J.N., Mishra, A., and Raskin, D.M. (2012). Stringent response regulation of biofilm formation in Vibrio cholerae. J. Bacteriol. *194*, 2962–2972.

Hirakawa, H., Inazumi, Y., Masaki, T., Hirata, T., and Yamaguchi, A. (2005). Indole induces the expression of multidrug exporter genes in Escherichia coli. Mol. Microbiol. *55*, 1113–1126.

Hoffman, L.R., D'Argenio, D.A., MacCoss, M.J., Zhang, Z., Jones, R.A., and Miller, S.I. (2005). Aminoglycoside antibiotics induce bacterial biofilm formation. Nature *436*, 1171–1175.

Høiby, N., Bjarnsholt, T., Givskov, M., Molin, S., and Ciofu, O. (2010). Antibiotic resistance of bacterial biofilms. Int. J. Antimicrob. Agents *35*, 322–332.

Junker, L.M., and Clardy, J. (2007). High-throughput screens for small-molecule inhibitors of Pseudomonas aeruginosa biofilm development. Antimicrob. Agents Chemother. *51*, 3582–3590.

Kayama, S., Murakami, K., Ono, T., Ushimaru, M., Yamamoto, A., Hirota, K., and Miyake, Y. (2009). The role of rpoS gene and quorum-sensing system in ofloxacin tolerance in Pseudomonas aeruginosa. FEMS Microbiol. Lett. *298*, 184–192.

Keren, I., Kaldalu, N., Spoering, A., Wang, Y., and Lewis, K. (2004). Persister cells and tolerance to antimicrobials. FEMS Microbiol. Lett. *230*, 13–18.

Keren, I., Wu, Y., Inocencio, J., Mulcahy, L.R., and Lewis, K. (2013). Killing by bactericidal antibiotics does not depend on reactive oxygen species. Science *339*, 1213–1216.

Kohanski, M.A., Dwyer, D.J., Hayete, B., Lawrence, C.A., and Collins, J.J. (2007). A common mechanism of cellular death induced by bactericidal antibiotics. Cell *130*, 797–810.

Kohanski, M.A., Dwyer, D.J., Wierzbowski, J., Cottarel, G., and Collins, J.J. (2008). Mistranslation of membrane proteins and two-component system activation trigger antibiotic-mediated cell death. Cell *135*, 679–690.

Kohanski, M.A., DePristo, M.A., and Collins, J.J. (2010). Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. Mol. Cell *37*, 311–320.

Korch, S.B., and Hill, T.M. (2006). Ectopic overexpression of wild-type and mutant hipA genes in Escherichia coli: effects on macromolecular synthesis and persister formation. J. Bacteriol. *188*, 3826–3836.

Korch, S.B., Henderson, T.A., and Hill, T.M. (2003). Characterization of the hipA7 allele of Escherichia coli and evidence that high persistence is governed by (p)ppGpp synthesis. Mol. Microbiol. *50*, 1199–1213.

Krause, P.J., Gewurz, B.E., Hill, D., Marty, F.M., Vannier, E., Foppa, I.M., Furman, R.R., Neuhaus, E., Skowron, G., Gupta, S., et al. (2008). Persistent and relapsing babesiosis in immunocompromised patients. Clin. Infect. Dis. *46*, 370–376.

Kucharíková, S., Tournu, H., Lagrou, K., Van Dijck, P., and Bujdáková, H. (2011). Detailed comparison of Candida albicans and Candida glabrata biofilms under different conditions and their susceptibility to caspofungin and anidulafungin. J. Med. Microbiol. *60*, 1261–1269.

Kussell, E., Kishony, R., Balaban, N.Q., and Leibler, S. (2005). Bacterial persistence: a model of survival in changing environments. Genetics *169*, 1807–1814.

LaCrue, A.N., Scheel, M., Kennedy, K., Kumar, N., and Kyle, D.E. (2011). Effects of artesunate on parasite recrudescence and dormancy in the rodent malaria model Plasmodium vinckei. PLoS ONE *6*, e26689.

LaFleur, M.D., Kumamoto, C.A., and Lewis, K. (2006). Candida albicans biofilms produce antifungal-tolerant persister cells. Antimicrob. Agents Chemother. *50*, 3839–3846.

Lafleur, M.D., Qi, Q., and Lewis, K. (2010). Patients with long-term oral carriage harbor high-persister mutants of Candida albicans. Antimicrob. Agents Chemother. *54*, 39–44.

Lee, S., Hinz, A., Bauerle, E., Angermeyer, A., Juhaszova, K., Kaneko, Y., Singh, P.K., and Manoil, C. (2009). Targeting a bacterial stress response to enhance antibiotic action. Proc. Natl. Acad. Sci. USA *106*, 14570–14575.

Lee, H.H., Molla, M.N., Cantor, C.R., and Collins, J.J. (2010). Bacterial charity work leads to population-wide resistance. Nature *467*, 82–85.

Leung, V., and Lévesque, C.M. (2012). A stress-inducible quorum-sensing peptide mediates the formation of persister cells with noninherited multidrug tolerance. J. Bacteriol. *194*, 2265–2274.

Levin, B.R., and Rozen, D.E. (2006). Non-inherited antibiotic resistance. Nat. Rev. Microbiol. *4*, 556–562.

Lieberman, T.D., Michel, J.B., Aingaran, M., Potter-Bynoe, G., Roux, D., Davis, M.R., Jr., Skurnik, D., Leiby, N., LiPuma, J.J., Goldberg, J.B., et al. (2011). Parallel bacterial evolution within multiple patients identifies candidate pathogenicity genes. Nat. Genet. *43*, 1275–1280.

Lin, G., Li, D., de Carvalho, L.P., Deng, H., Tao, H., Vogt, G., Wu, K., Schneider, J., Chidawanyika, T., Warren, J.D., et al. (2009). Inhibitors selective for mycobacterial versus human proteasomes. Nature *461*, 621–626.

Liu, Y., and Imlay, J.A. (2013). Cell death from antibiotics without the involvement of reactive oxygen species. Science *339*, 1210–1213.

Lu, T.K., and Collins, J.J. (2007). Dispersing biofilms with engineered enzymatic bacteriophage. Proc. Natl. Acad. Sci. USA *104*, 11197–11202.

Lu, T.K., and Collins, J.J. (2009). Engineered bacteriophage targeting gene networks as adjuvants for antibiotic therapy. Proc. Natl. Acad. Sci. USA *106*, 4629–4634.

Maisonneuve, E., Shakespeare, L.J., Jørgensen, M.G., and Gerdes, K. (2011). Bacterial persistence by RNA endonucleases. Proc. Natl. Acad. Sci. USA *108*, 13206–13211.

McCune, R.M., Jr., and Tompsett, R. (1956). Fate of Mycobacterium tuberculosis in mouse tissues as determined by the microbial enumeration technique. I. The persistence of drug-susceptible tubercle bacilli in the tissues despite prolonged antimicrobial therapy. J. Exp. Med. *104*, 737–762.

McCune, R.M., Jr., McDermott, W., and Tompsett, R. (1956). The fate of Mycobacterium tuberculosis in mouse tissues as determined by the microbial enumeration technique. II. The conversion of tuberculous infection to the latent state by the administration of pyrazinamide and a companion drug. J. Exp. Med. *104*, 763–802.

Miotto, O., Almagro-Garcia, J., Manske, M., Macinnis, B., Campino, S., Rockett, K.A., Amaratunga, C., Lim, P., Suon, S., Sreng, S., et al. (2013). Multiple populations of artemisinin-resistant Plasmodium falciparum in Cambodia. Nat. Genet. *45*, 648–655. http://dx.doi.org/10.1038/ng.2624.

Möker, N., Dean, C.R., and Tao, J. (2010). Pseudomonas aeruginosa increases formation of multidrug-tolerant persister cells in response to quorum-sensing signaling molecules. J. Bacteriol. *192*, 1946–1955.

Moyed, H.S., and Bertrand, K.P. (1983). hipA, a newly recognized gene of Escherichia coli K-12 that affects frequency of persistence after inhibition of murein synthesis. J. Bacteriol. *155*, 768–775.

Murakami, K., Ono, T., Viducic, D., Kayama, S., Mori, M., Hirota, K., Nemoto, K., and Miyake, Y. (2005). Role for rpoS gene of Pseudomonas aeruginosa in antibiotic tolerance. FEMS Microbiol. Lett. *242*, 161–167.

Mwangi, M.M., Wu, S.W., Zhou, Y., Sieradzki, K., de Lencastre, H., Richardson, P., Bruce, D., Rubin, E., Myers, E., Siggia, E.D., and Tomasz, A. (2007). Tracking the in vivo evolution of multidrug resistance in Staphylococcus aureus by whole-genome sequencing. Proc. Natl. Acad. Sci. USA *104*, 9451–9456.

Nair, C.G., Chao, C., Ryall, B., and Williams, H.D. (2013). Sub-lethal concentrations of antibiotics increase mutation frequency in the cystic fibrosis pathogen Pseudomonas aeruginosa. Lett. Appl. Microbiol. *56*, 149–154.

Nakazawa, S., Kanbara, H., and Aikawa, M. (1995). Plasmodium falciparum: recrudescence of parasites in culture. Exp. Parasitol. *81*, 556–563.

Nakazawa, S., Maoka, T., Uemura, H., Ito, Y., and Kanbara, H. (2002). Malaria parasites giving rise to recrudescence in vitro. Antimicrob. Agents Chemother. *46*, 958–965.

Nguyen, D., Joshi-Datar, A., Lepine, F., Bauerle, E., Olakanmi, O., Beer, K., McKay, G., Siehnel, R., Schafhauser, J., Wang, Y., et al. (2011). Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. Science *334*, 982–986.

Perriëns, J.H., St Louis, M.E., Mukadi, Y.B., Brown, C., Prignot, J., Pouthier, F., Portaels, F., Willame, J.C., Mandala, J.K., Kaboto, M., et al. (1995). Pulmonary tuberculosis in HIV-infected patients in Zaire. A controlled trial of treatment for either 6 or 12 months. N. Engl. J. Med. *332*, 779–784.

Petrosino, J.F., Galhardo, R.S., Morales, L.D., and Rosenberg, S.M. (2009). Stress-induced beta-lactam antibiotic resistance mutation and sequences of stationary-phase mutations in the Escherichia coli chromosome. J. Bacteriol. *191*, 5881–5889.

Pettit, R.K., Repp, K.K., and Hazen, K.C. (2010). Temperature affects the susceptibility of Cryptococcus neoformans biofilms to antifungal agents. Med. Mycol. *48*, 421–426.

Poole, K. (2012). Stress responses as determinants of antimicrobial resistance in Gram-negative bacteria. Trends Microbiol. *20*, 227–234.

Potrykus, K., and Cashel, M. (2008). (p)ppGpp: still magical? Annu. Rev. Microbiol. *62*, 35–51.

Prudhomme, M., Attaiech, L., Sanchez, G., Martin, B., and Claverys, J.P. (2006). Antibiotic stress induces genetic transformability in the human pathogen Streptococcus pneumoniae. Science *313*, 89–92.

Rovira-Graells, N., Gupta, A.P., Planet, E., Crowley, V.M., Mok, S., Ribas de Pouplana, L., Preiser, P.R., Bozdech, Z., and Cortés, A. (2012). Transcriptional variation in the malaria parasite Plasmodium falciparum. Genome Res. *22*, 925–938.

Ryder, V.J., Chopra, I., and O'Neill, A.J. (2012). Increased mutability of Staphylococci in biofilms as a consequence of oxidative stress. PLoS ONE *7*, e47695.

Saralamba, S., Pan-Ngum, W., Maude, R.J., Lee, S.J., Tarning, J., Lindegårdh, N., Chotivanich, K., Nosten, F., Day, N.P., Socheat, D., et al. (2011). Intrahost modeling of artemisinin resistance in Plasmodium falciparum. Proc. Natl. Acad. Sci. USA *108*, 397–402.

Savage, V.J., Chopra, I., and O'Neill, A.J. (2013). Staphylococcus aureus biofilms promote horizontal transfer of antibiotic resistance. Antimicrob. Agents Chemother. *57*, 1968–1970.

Shah, D., Zhang, Z., Khodursky, A., Kaldalu, N., Kurg, K., and Lewis, K. (2006). Persisters: a distinct physiological state of E. coli. BMC Microbiol. *6*, 53.

Sharma, S.V., Lee, D.Y., Li, B., Quinlan, M.P., Takahashi, F., Maheswaran, S., McDermott, U., Azizian, N., Zou, L., Fischbach, M.A., et al. (2010). A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. Cell *141*, 69–80.

Shatalin, K., Shatalina, E., Mironov, A., and Nudler, E. (2011). H2S: a universal defense against antibiotics in bacteria. Science *334*, 986–990.

Spoering, A.L., Vulic, M., and Lewis, K. (2006). GlpD and PlsB participate in persister cell formation in Escherichia coli. J. Bacteriol. *188*, 5136–5144.

Stecher, B., Denzler, R., Maier, L., Bernet, F., Sanders, M.J., Pickard, D.J., Barthel, M., Westendorf, A.M., Krogfelt, K.A., Walker, A.W., et al. (2012). Gut inflammation can boost horizontal gene transfer between pathogenic and commensal Enterobacteriaceae. Proc. Natl. Acad. Sci. USA *109*, 1269–1274.

Stevens, K.E., Chang, D., Zwack, E.E., and Sebert, M.E. (2011). Competence in Streptococcus pneumoniae is regulated by the rate of ribosomal decoding errors. MBio. *2*, e00071–11.

Sun, G., Luo, T., Yang, C., Dong, X., Li, J., Zhu, Y., Zheng, H., Tian, W., Wang, S., Barry, C.E., 3rd., et al. (2012). Dynamic population changes in Mycobacterium tuberculosis during acquisition and fixation of drug resistance in patients. J. Infect. Dis. *206*, 1724–1733.

Takala-Harrison, S., Clark, T.G., Jacob, C.G., Cummings, M.P., Miotto, O., Dondorp, A.M., Fukuda, M.M., Nosten, F., Noedl, H., Imwong, M., et al. (2013). Genetic loci associated with delayed clearance of Plasmodium

falciparum following artemisinin treatment in Southeast Asia. Proc. Natl. Acad. Sci. USA *110*, 240–245.

Teuscher, F., Gatton, M.L., Chen, N., Peters, J., Kyle, D.E., and Cheng, Q. (2010). Artemisinin-induced dormancy in plasmodium falciparum: duration, recovery rates, and implications in treatment failure. J. Infect. Dis. *202*, 1362–1368.

Thapar, M.M., Gil, J.P., and Björkman, A. (2005). In vitro recrudescence of Plasmodium falciparum parasites suppressed to dormant state by atovaquone alone and in combination with proguanil. Trans. R. Soc. Trop. Med. Hyg. *99*, 62–70.

Tripathi, A., Dewan, P.C., Barua, B., and Varadarajan, R. (2012). Additional role for the ccd operon of F-plasmid as a transmissible persistence factor. Proc. Natl. Acad. Sci. USA *109*, 12497–12502.

Van Acker, H., Sass, A., Bazzini, S., De Roy, K., Udine, C., Messiaen, T., Riccardi, G., Boon, N., Nelis, H.J., Mahenthiralingam, E., and Coenye, T. (2013). Biofilm-grown Burkholderia cepacia complex cells survive antibiotic treatment by avoiding production of reactive oxygen species. PLoS ONE *8*, e58943.

Vega, N.M., Allison, K.R., Khalil, A.S., and Collins, J.J. (2012). Signaling-mediated bacterial persister formation. Nat. Chem. Biol. *8*, 431–433.

Wakamoto, Y., Dhar, N., Chait, R., Schneider, K., Signorino-Gelo, F., Leibler, S., and McKinney, J.D. (2013). Dynamic persistence of antibiotic-stressed mycobacteria. Science *339*, 91–95.

Wang, X., and Zhao, X. (2009). Contribution of oxidative damage to antimicrobial lethality. Antimicrob. Agents Chemother. *53*, 1395–1402.

Wexselblatt, E., Oppenheimer-Shaanan, Y., Kaspy, I., London, N., Schueler-Furman, O., Yavin, E., Glaser, G., Katzhendler, J., and Ben-Yehuda, S. (2012). Relacin, a novel antibacterial agent targeting the Stringent Response. PLoS Pathog. *8*, e1002925.

White, N.J., Pongtavornpinyo, W., Maude, R.J., Saralamba, S., Aguas, R., Stepniewska, K., Lee, S.J., Dondorp, A.M., White, L.J., and Day, N.P. (2009). Hyperparasitaemia and low dosing are an important source of anti-malarial drug resistance. Malar. J. *8*, 253.

Wigle, T.J., Sexton, J.Z., Gromova, A.V., Hadimani, M.B., Hughes, M.A., Smith, G.R., Yeh, L.A., and Singleton, S.F. (2009). Inhibitors of RecA activity discovered by high-throughput screening: cell-permeable small molecules attenuate the SOS response in Escherichia coli. J. Biomol. Screen. *14*, 1092–1101.

Witkowski, B., Lelièvre, J., Barragán, M.J., Laurent, V., Su, X.Z., Berry, A., and Benoit-Vical, F. (2010). Increased tolerance to artemisinin in Plasmodium falciparum is mediated by a quiescence mechanism. Antimicrob. Agents Chemother. *54*, 1872–1877.

Wu, Y., Vulić, M., Keren, I., and Lewis, K. (2012). Role of oxidative stress in persister tolerance. Antimicrob. Agents Chemother. *56*, 4922–4926.

Zhang, X., McDaniel, A.D., Wolf, L.E., Keusch, G.T., Waldor, M.K., and Acheson, D.W. (2000). Quinolone antibiotics induce Shiga toxin-encoding bacteriophages, toxin production, and death in mice. J. Infect. Dis. *181*, 664–670.

Zhang, Q., Lambert, G., Liao, D., Kim, H., Robin, K., Tung, C.K., Pourmand, N., and Austin, R.H. (2011). Acceleration of emergence of bacterial antibiotic resistance in connected microenvironments. Science *333*, 1764–1767.

Zhao, J., Schloss, P.D., Kalikin, L.M., Carmody, L.A., Foster, B.K., Petrosino, J.F., Cavalcoli, J.D., VanDevanter, D.R., Murray, S., Li, J.Z., et al. (2012). Decade-long bacterial community dynamics in cystic fibrosis airways. Proc. Natl. Acad. Sci. USA *109*, 5809–5814.