Cell

Leading Edge
Review

Targeting Antibiotic Tolerance, Pathogen by Pathogen

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https://doi.org/10.1016/j.cell.2018.01.037

Antibiotic tolerance, the capacity of genetically susceptible bacteria to survive the lethal effects of antibiotic treatment, plays a critical and underappreciated role in the disease burden of bacterial infections. Here, we take a pathogen-by-pathogen approach to illustrate the clinical significance of antibiotic tolerance and discuss how the physiology of specific pathogens in their infection environments impacts the mechanistic underpinnings of tolerance. We describe how these insights are leading to the development of species-specific therapeutic strategies for targeting antibiotic tolerance and highlight experimental platforms that are enabling us to better understand the complexities of drug-tolerant pathogens in *in vivo* settings.

Introduction

Antibiotic resistance is now widely recognized as a threat to the efficacy of our antibiotic arsenal. In the United States alone, an estimated 2 million infections and 23,000 deaths occur each year due to antibiotic-resistant pathogens (CDC, 2013). However, genetically encoded resistance is not the only mechanism that bacteria can use to withstand antibiotic therapy. Indeed, bacteria that are genetically susceptible, yet phenotypically tolerant to treatment, play a critical role in perpetuating chronic and recurrent infections (Fauvart et al., 2011; Grant and Hung, 2013). Furthermore, recent *in vitro* evidence supports the hypothesis that antibiotic tolerance can also cause treatment failure by facilitating the acquisition of antibiotic resistance (Cohen et al., 2013; Levin-Reisman et al., 2017).

Antibiotic tolerance has been defined, and is quantified *in vitro*, using a number of different metrics that describe the time-dependent and concentration-dependent lethality of antibiotics (Brauner et al., 2016). In this review, we will utilize a qualitative definition of tolerance due to our limited understanding of how *in vitro* observations of antibiotic tolerance pertain to the treatment of *in vivo* infections. Here, we consider an antibiotic-tolerant infection to be synonymous with an antibiotic-persistent infection (Grant and Hung, 2013) and to be characterized by the presence of a pathogen that is susceptible to antibiotics by standard microbiology lab assays yet is not cleared by antimicrobial therapy. *In vitro* studies that report "antibiotic tolerance" or "antibiotic persistence"—phenotypes that are characterized by a reduction in antibiotic lethality—will be interpreted here to describe potential mechanisms that may contribute to antibiotic-tolerant infections *in vivo*.

In the past decade, progress has been made in identifying the physiologic and genetic determinants of antibiotic-tolerant cell states (Van den Bergh et al., 2017). In parallel, research has advanced our understanding of the context-dependent mechanisms underlying antibiotic lethality (Yang et al., 2017a). The insights gained from these research efforts are fundamentally interlinked. Antibiotic entry into the cell, corruption of target activity, and perturbations of downstream metabolic processes determine cell fate under antibiotic treatment. Tolerance-conferring effects, mediated by cellular stress responses and related systems, are induced by a combination of environmental stresses and genetic factors that are translated into physiological alterations in bacteria that disrupt lethal events triggered by bactericidal antibiotics.

In this review, we take a pathogen-by-pathogen approach to provide a distinctly different perspective on antibiotic tolerance and to highlight its clinical significance. While prior commentaries have constructed comprehensive lists of bacteria that are prone to forming antibiotic-tolerant infections (Cohen et al., 2013; Grant and Hung, 2013), we focus here on four pathogens: *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis*. For each pathogen, we start with a representative case—inspired by infectious disease clinical consultations—where drug tolerance critically influences the course of treatment. This is followed by a presentation of evidence that demonstrates a role for antibiotic tolerance in leading to treatment failure and the associated broad epidemiological consequences of infections caused by the pathogen. In each case, we then describe a selection of emerging strategies for targeting these antibiotic-tolerant infections that build on

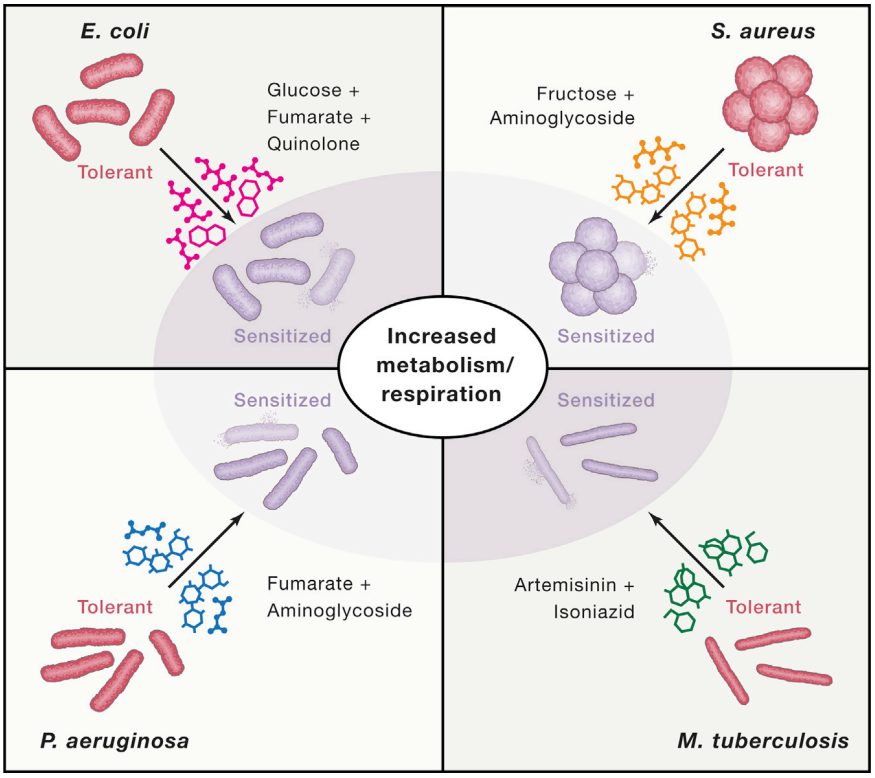

Figure 1. Metabolism-Directed Strategies for Targeting Antibiotic Tolerance

Shifting an antibiotic-tolerant pathogen into a metabolically susceptible state is a promising approach for treating tolerance. This general strategy must be tailored on a pathogen-by-pathogen basis, however, to be successful. Here, we show how addition of a metabolic stimulus results in sensitization of the pathogen to antibiotic lethality. For *E.* coli, addition of glucose and fumarate stimulates respiratory metabolism and sensitizes the bacterial population to quinolone lethality. Addition of fructose and fumarate stimulates an increase in proton motive force that results in increased aminoglycoside uptake and lethality in *S. aureus* and *P. aeruginosa*, respectively. Isoniazid efficacy is restored via the chemical inhibition by artemisinin of the dormancy-inducing *M. tuberculosis* DosRST regulon.

mechanistic insights into tolerance. The pathogen-by-pathogen analysis allows for an appreciation for how both the biology of the pathogen and the environmental context of infection impact the efficacy of drug treatments. This approach reveals that there are generalizable strategies for targeting antibiotic tolerance that require specific modifications and implementations on a pathogen-by-pathogen basis (Figure 1). While impressive, these and other therapeutic approaches would benefit from additional, clinically meaningful insights into antibiotic tolerance. Thus, we also highlight vital research efforts to develop experimental tools to enable a better understanding of antibiotic tolerance in *in vivo* settings.

Pathogen Portraits of Tolerance

Escherichia coli

Patient E.C. is 87 years old with an indwelling catheter and resides in a long-term care facility. She develops a fever and lower abdominal pain and is diagnosed with a susceptible E. coli *urinary tract infection. Multiple attempts to exchange her indwelling catheter are unsuccessful and thus the catheter is left in situ. Shortly after receiving a 2-week course of antibiotics without urinary catheter replacement, the infection relapses, triggering a readmission to the hospital.*

The case of Patient E.C. exemplifies the role that antibiotic tolerance plays in perpetuating the clinical burden of urinary tract infections (UTIs). Globally, UTIs impact 150 million people per year, leading to an economic burden of $3.5 billion in the United States alone (Flores-Mireles et al., 2015). Approximately 75% of these infections are due to uropathogenic *E. coli* (UPEC) and 70%–80% of complicated UTIs are catheter-associated (Flores-Mireles et al., 2015). In a catheter-associated UTI, an antibiotic-tolerant biofilm is formed via adherence to the surface of the catheter (Jacobsen et al., 2008), and treatment of this condition usually requires catheter replacement even if the pathogen is genetically susceptible (Hooton et al., 2010).

E. coli UTIs are frequently formed due to the translocation of gut strains to the urinary tract (Flores-Mireles et al., 2015). This transition from the diverse nutritional environment of the gut to the nutrient-poor, nitrogen-rich, high-osmolarity environment of the urinary tract is associated with the activation of tolerance-conferring stress responses, such as the stringent response (Aberg et al., 2006; Alteri and Mobley, 2015). Stress response activation induces the alteration of multiple cellular processes, including lowering the proton-motive force (PMF) (Verstraeten et al., 2015), decreasing cell wall turnover (Rodionov and Ishiguro, 1996), altering DNA replication and repair systems such as the SOS response (Durfee et al., 2008), inducing biofilm formation, and establishing intracellular bacterial communities (Aberg et al., 2006). The net effect is a physiological state that is unfavorable to the lethal actions of major classes of antibiotics (Bernier et al., 2013; Ito et al., 2009; Verstraeten et al., 2015). In particular, the formation of biofilms can induce the appearance of a subpopulation of quasi-dormant cells that challenge bactericidal treatments that rely on active, energy-dependent processes (Ito et al., 2009).

One strategy to potentiate antibiotic lethality in these conditions is to modify the pathogen's metabolic environment so as to revert these tolerance-conferring effects. In an early demonstration of this strategy, a screen of select carbon sources from glycolysis, the pentose-phosphate pathway, and the Entner-Doudoroff pathway, identified that addition of pyruvate, mannitol, fructose, or glucose, resulted in potentiation of aminoglycoside lethality towards antibiotic-tolerant *E. coli* populations (Allison et al., 2011). Upon addition to the extracellular environment, these metabolites are transported to the cytoplasm and enter glycolysis, where their catabolism generates NADH. This NADH is

then oxidized by enzymes in the electron transport chain (ETC), which contributes to an increased PMF that facilitates uptake of aminoglycosides. This adjuvant strategy was shown to be effective in treating *E. coli* biofilms *in vitro*, as well as in a murine catheter-associated biofilm UTI model (Allison et al., 2011).

Sensitization of tolerant *E. coli* to aminoglycosides can also be achieved by altering the environmental pH of the infection site. Lebeaux and coworkers reported that the lethality of aminoglycosides towards antibiotic-tolerant *E. coli* could be recovered by increasing the environmental pH via supplementation with the basic amino acid, L-arginine, resulting in an increased PMF and subsequent drug uptake (Lebeaux et al., 2014). This strategy was also successfully tested *in vivo*, where it enabled the eradication of an *E. coli* catheter-associated infection in a murine model (Lebeaux et al., 2014). Additionally, a recent study demonstrated that bicarbonate, an essential component of the bicarbonate buffering system in human tissue, has pH-mediated effects on PMF that enhance the susceptibility of *E. coli* to aminoglycosides (Farha et al., 2018).

The effectiveness of metabolite-based potentiation strategies is not limited to aminoglycosides. Gutierrez and colleagues have shown that metabolic stimulation can also successfully sensitize antibiotic-tolerant cells to quinolones like ciprofloxacin (Gutierrez et al., 2017). In this study, the authors demonstrated that external limits to specific metabolic pathways, rather than cellular responses to starvation, are the key factors modulating density-dependent-persistence to quinolones. It was also shown that when carbon sources are limiting, replenishment of carbon oxidation pathways alone is insufficient for quinolone activity and must be accompanied by a suitable terminal electron acceptor.

Using this framework, the authors demonstrated that quinolone lethality towards tolerant, stationary phase cultures of *E. coli* could be enhanced via the addition of glucose as the carbon source and fumarate or oxygen as the terminal electron acceptor (Figure 1) (Gutierrez et al., 2017). In support of the proposed mechanism, sensitization was attenuated when the bacteria were rendered genetically incapable of utilizing the specifically added terminal electron acceptor. Given that drug potentiation was observed to be independent of cell growth, this study highlights the importance of respiratory metabolism for determining the lethality of quinolone treatment.

An alternative strategy to perturbing the metabolic environment of an infection site is the use of engineered vectors such as bacteriophages and phagemids to enable the targeted delivery of genetic components that act to reprogram the physiological state of the pathogen. In contrast to the use of bacteriophages that deliver lethal genes, a treatment strategy that combines phage delivery of network-altering genes with antibiotic treatment is less likely to suffer from phage resistance (Lu and Collins, 2009). The targeting of stress pathways such as the SOS response enabled increased antibiotic efficacy for a range of bactericidal antibiotics against *E. coli* persisters and antibiotic-tolerant *E. coli* biofilms (Lu and Collins, 2009). Furthermore, phage therapy prevented the development of resistant mutants at sub-inhibitory concentrations of antibiotics (Lu and Collins, 2009). Such a platform is not limited to the down-regulation of the SOS response and could carry synthetic gene circuits that modulate aforementioned tolerance-conferring pathways (e.g., csrA, a biofilm repressor) or other adjuvant genes (e.g., porins such as ompF) (Lu and Collins, 2009). In contrast, the use of engineered phagemids to express antimicrobial peptides or toxins has been shown to be effective without needing concurrent administration with antibiotics and is also effective at avoiding challenges with phage resistance (Krom et al., 2015). Crucially, both engineered bacteriophages and phagemids have been validated as therapeutic strategies in murine infection models (Krom et al., 2015; Lu and Collins, 2009).

Staphylococcus aureus

Patient S.A. is 54 and underwent hip replacement 3 weeks ago. He has developed pain and redness at the surgical site. Sampling of the hip reveals methicillin-susceptible S. aureus. *Despite this susceptibility, antibiotic therapy is unable to clear his infection. Patient S.A. will require surgery in addition to a course of antibiotics lasting multiple weeks.*

In the United States alone, approximately 1 million knee and hip joint replacement surgeries are performed every year, of which roughly 2% will be complicated by a bacterial infection resulting in an economic burden exceeding $500 million (Tande and Patel, 2014). Up to 30% of these infections are attributed to *S. aureus* (Tande and Patel, 2014; Zimmerli et al., 2004). The formation of biofilms on the surface of the prosthesis is a key mechanism that enables antibiotic tolerance in the context of prosthetic joint infections (Zimmerli et al., 2004). Protracted infections with *S. aureus* have also been linked to pathogens surviving in host cells (Gresham et al., 2000). For example, antibiotic-tolerant intracellular forms of *S. aureus* are thought to contribute to the persistence of *S. aureus* bacteremia (Gresham et al., 2000; Lehar et al., 2015). The ability of these intracellular forms to survive antibiotic treatment facilitates the establishment of metastatic foci of infection (Gresham et al., 2000).

When converting to a biofilm, *S. aureus* produces an extracellular matrix and undergoes changes in physiology that include a decrease in cellular respiration, protein synthesis, and DNA replication (Rani et al., 2007). *S. aureus* can also adopt a small colony variant (SCV) phenotype in response to certain aspects of the environment of a prosthetic joint infection, including nutrient starvation and exposure to antimicrobial and host cationic peptides (Proctor et al., 2014; Sendi et al., 2006). This SCV phenotype is characterized by a decrease in cellular metabolism (Sendi and Proctor, 2009) and a transition from oxidative respiration utilizing the tricarboxylic acid (TCA) cycle to arginine deamination (Proctor et al., 2014), leading to decreased cellular respiration and PMF. Similarly, intracellular forms of *S. aureus* encounter an acidic, nutrient-starved environment along with exposure to oxidative stress and adopt an SCV phenotype (Sendi and Proctor, 2009). Similar to other bacteria, an increased presence of free toxins from toxin-antitoxin modules results in slowed growth, and is linked to persistence. For *S. aureus*, overexpression of the toxin MazF and/or the proteolytic degradation of the cognate antitoxin MazE by ClpP results in the adoption of an SCV phenotype (Proctor et al., 2014).

In treating *S. aureus*, antibiotic tolerance is again characterized by an induction of tolerance-conferring cellular processes. Importantly, these processes can either be circumvented or directly targeted using appropriately designed therapies. Pentobra, for example, is a new aminoglycoside designed specifically

to circumvent the antibiotic tolerance that is conferred by low-energy cell states (Schmidt et al., 2014). The premise of this antimicrobial development strategy is the addition of a 12-peptide component to the aminoglycoside moiety that enables energy-independent uptake by the cell, bypassing the PMF-dependency that is typically observed with aminoglycosides. Pentobra has been shown *in vitro* to have significantly increased lethality towards antibiotic-tolerant *S. aureus* cell populations (Schmidt et al., 2014). In contrast, ClpP activation showcases an example of directly targeting tolerance-conferring cellular processes in *S. aureus* infections (Conlon et al., 2013). ADEP4, amongst other substrates, was identified to cause the nonspecific activation of the ClpP protease independently of the cognate ATPase (Brötz-Oesterhelt et al., 2005). Combination treatment of ADEP4 with rifampicin was shown to have a potent effect *in vitro* against *S. aureus* biofilms and *in vivo* in a murine thigh infection model (Conlon et al., 2013).

Metabolite-based sensitization strategies have also been shown to be effective in treating antibiotic-tolerant *S. aureus* (Allison et al., 2011; Gutierrez et al., 2017). However, it is interesting to note that these strategies must be tuned on a pathogen-by-pathogen basis to be successful. Of the four metabolites used to potentiate aminoglycoside efficacy in *E. coli* (glucose, fructose, mannitol, pyruvate), fructose was the only one that increased the efficacy of gentamicin treatment of *S. aureus* biofilms (Figure 1) (Allison et al., 2011). Based on expression analysis, this was shown to be due to differential expression of metabolite transporters in growing versus dormant *S. aureus*. In the case of quinolone potentiation in high-density cultures, supplementation of oxygen and glucose was able to increase the efficacy of quinolone treatment of antibiotic-tolerant *S. aureus*, while fumarate could not be used since *S. aureus* is unable to use fumarate as an alternative electron acceptor (Gutierrez et al., 2017).

An independent but synergistic strategy is to concentrate the antibacterial therapy at the site of interest. In the case of *S. aureus,* intracellular bacteria can be targeted by locally increasing the concentration of antibiotics around the host cells. Mariathasan and coworkers conjugated a rifamycin analog to a targeting antibody with a cathepsin-cleavable bridge (Lehar et al., 2015). This enabled the destruction of phagocytosed *S. aureus* cells with impressive efficacy in both *in vitro* and *in vivo* experiments. However, it should be noted that the effectiveness of the antibody-antibiotic complex was only tested in administrations that were given up to 24 hours after infection. This treatment schedule contrasts with a clinical setting where the intracellular bacteria may have been internalized for a much longer period of time; further testing is thus needed to determine the clinical efficacy of this strategy.

Pseudomonas aeruginosa

Patient P.A. is 35 and has cystic fibrosis. Her lungs have been chronically infected with P. aeruginosa *for years and although inhaled tobramycin has helped, it has not eradicated the pathogen. As a result, Patient P.A.'s worsening pulmonary function makes her a candidate for pulmonary transplant.*

Due to the altered physiology of airways in the lungs of cystic fibrosis (CF) patients, Patient P.A. had a significantly increased risk for the development of a chronic lung infection with *P. aeruginosa*. Eighty percent of CF patients develop a chronic *P. aeruginosa* pulmonary infection, and such infections are the single strongest predictors of morbidity and mortality for CF patients (O'Sullivan and Freedman, 2009). Of note, *P. aeruginosa* infections are often characterized by biofilm-induced antibiotic tolerance. Analyses of explanted lungs from affected patients have revealed aggregates, confirming the presence of biofilms, and a notable level of metabolic diversity within the bacterial community (Jorth et al., 2015). High-dose aminoglycoside treatment of chronic infections for extended periods of time in CF patients does not eradicate the pathogen. At best, this treatment strategy decreases the bacterial burden in the infection, albeit with a progressive decline in efficacy over time (Ramsey et al., 1999). Importantly, the genetic susceptibility of clinical isolates from these infections can remain unchanged, supporting the notion that antibiotic tolerance plays a role in treatment failure (Ramsey et al., 1999).

The local environment in the airways of a CF patient is marked by desiccated mucus, low oxygen, and perpetual inflammation, which all influence the physiology of *P. aeruginosa*. This environment pushes the pathogen to form biofilms, most likely through the combined effect of the stringent response, quorum sensing, and cyclic di-guanosine monophosphate (c-di-GMP) (Hazan et al., 2016; Malone et al., 2010; Nguyen et al., 2011; Singh et al., 2000). Quorum sensing is known to down-regulate metabolism (Davenport et al., 2015), reduce DNA replication (Withers and Nordström, 1998), up-regulate efflux pump activity (Maseda et al., 2004), and participate in biofilm formation (Hazan et al., 2016; Jakobsen et al., 2017). High levels of quorum sensing molecules are consistently seen in the sputum of CF patients that are chronically infected with *P. aeruginosa* (Singh et al., 2000). Additionally, the production of c-di-GMP is triggered by the decreased nutrient availability at the site of infection (Basu Roy and Sauer, 2014) and has been identified as a key second messenger that controls the cellular transition into a biofilm state (Jenal et al., 2017). In particular, c-di-GMP is associated with antibiotic-tolerant *P. aeruginosa* SCVs that are documented to overproduce extracellular matrix in CF patients (Malone et al., 2010).

Quorum sensing molecules and c-di-GMP are clearly involved in inducing physiological changes in *P. aeruginosa* that are unfavorable to antibiotic lethality. As a result, they are promising targets for reprogramming tolerant cells towards a more drug-susceptible state. There are several candidate classes of quorum sensing inhibitors (QSIs) under development. In the case of *P. aeruginosa*, candidates include bromo-furanones, patulin, penicillic acid, ajoene, mBTL, and benzamide-benzimidazole (Brackman and Coenye, 2015; O'Loughlin et al., 2013; Starkey et al., 2014). Inhibition of quorum sensing has been shown to induce a dispersion of *P. aeruginosa* biofilms (Hentzer et al., 2003) and increase sensitivity to aminoglycosides in *in vivo P. aeruginosa* experiments (Christensen et al., 2012; Maura and Rahme, 2017). However, current QSIs target downstream regulators as opposed to the central regulators of quorum sensing, which likely accounts for their mild to moderate efficacy (Manefield et al., 2002). It is also relevant to note that resistance to QSIs has been observed, which presents an additional challenge to overcome in the development and implementation of these antibacterials (Maeda et al., 2012).

Inhibitors of c-di-GMP have been identified and proven efficacious in disrupting and dispersing *P. aeruginosa* biofilms (Sambanthamoorthy et al., 2014), though antibiotic potentiation was not tested in these studies. Enhancing c-di-GMP inactivating enzymes is a complementary approach to inactivating the pathway. Howlin and coworkers showed that nitric oxide, a phosphodiesterase activator, could disrupt *P. aeruginosa* biofilms and thereby potentiate antibiotic efficacy (Howlin et al., 2017). In the same study, treatment of patients with antibiotics in combination with nitric oxide resulted in increased biofilm dispersion; however, a decrease in bacterial burden in patient sputum or improved respiratory function was not observed. The pleiotropic effects of nitric oxide make it difficult to interpret this pilot study, and further work is needed to determine and potentially enhance the clinical efficacy of this promising approach.

Potentiation of aminoglycoside lethality in metabolically quasi-dormant cells with the addition of specific metabolites has also been demonstrated in *P. aeruginosa*. In a recent study, a metabolite screen conducted with carbon sources from central metabolism pathways identified a set of metabolites that could potentiate tobramycin lethality towards tolerant, stationary phase cultures of *P. aeruginosa* (Meylan et al., 2017). Focusing on one of these metabolites, the authors found that supplementation with fumarate—a lower TCA cycle metabolite—promoted TCA cycle activity, which produced reduced electron carriers to fuel the ETC and increase PMF-dependent uptake of tobramycin (Figure 1). In this same study, glyoxylate—an upper TCA cycle metabolite—was shown to suppress cellular respiration and provide protection against tobramycin treatment. Interestingly, glyoxylate acted as a dominant negative inducer of antibiotic tolerance over fumarate potentiation and this phenotype was observed despite no apparent effect on membrane potential or aminoglycoside uptake. This finding suggests that uptake may not be sufficient for aminoglycoside lethality and that there exists a more important role than conventionally thought for downstream metabolic processes in determining the efficacy of aminoglycoside treatment. The fumarate supplementation strategy has also been shown to be effective in the potentiation of tobramycin treatment of *P. aeruginosa* clinical isolates from CF patients, in a range of environments aimed at assessing the effectiveness of this strategy in clinical scenarios (Koeva et al., 2017). Together, these studies indicate that a combination of tobramycin and fumarate may be an attractive strategy for treating tolerant *P. aeruginosa* infections in CF patients.

Mycobacterium tuberculosis

Patient M.T. is 37 and has experienced coughing and weight loss for 3 months. On a sputum exam, he is found to have a fully susceptible M. tuberculosis *infection. His doctor informs him that he will require at least 6 months of therapy, of which the first 2 months will require four antibiotics. Due to toxicity related to the drugs, Patient M.T. will have to undergo regular check-ups.*

Approximately 9 million people per year experience an active infection with *M. tuberculosis*, leading to 1.5 million deaths (Horsburgh et al., 2015). Furthermore, there exists a latent reservoir of *M. tuberculosis*-infected individuals that has been estimated to include roughly a third of the world's population (Getahun et al., 2015). *M. tuberculosis* is one of the most striking clinical examples of a tolerant infection, as effective treatment necessitates a 6-month multidrug regimen (Horsburgh et al., 2015). Anti-tuberculosis therapy development has been marked by the failure of single-agent therapies to achieve a cure with a repeated pattern of an initial treatment response followed by relapse, due to the development of resistant strains (Fox et al., 1957). While the development of combination therapies has improved clinical outcomes, the persistence of bacilli in patients despite combination therapies and associated high treatment failure rates necessitate prolonged treatment periods (Horsburgh et al., 2015). Here again, tolerance has been linked to the heterogeneous environment at the site of infection, with intracellular subpopulations and cells in granulomas displaying particularly decreased drug susceptibility (Wayne and Sohaskey, 2001).

The propensity of *M. tuberculosis* to adopt a metabolically dormant state has been linked to the tolerance of the pathogen to antimicrobial therapy. In particular, the response of gene regulatory networks within the pathogen to diminished oxygen and lipid availability is an important mediator of the transition to dormancy (Galagan et al., 2013). In this transition, different regulatory networks have been shown to respond on distinct time-scales. For example, hypoxic conditions induce an initial response that is mediated in part by the DosRST regulon, which is followed by the enduring hypoxic response stimulon at later time points (Galagan et al., 2013). Through the DosRST regulon, *M. tuberculosis* can sense hypoxia in a caseating granuloma as well as the presence of nitrosative stress in a lysosome (Liu et al., 2016). This two-component system oversees the transcriptional regulation of 50 genes that govern a decrease in cellular metabolism and entry into a non-replicating persistent state (Galagan et al., 2013; Voskuil et al., 2003). The up-regulation of the DosRST regulon in response to the stress imposed by active host responses has been consistently shown to correlate with antibiotic tolerance in tissue culture and in *in vivo* models of *M. tuberculosis* infection (Liu et al., 2016).

In an effort to counteract this transition to dormancy, Abramovitch and coworkers recently identified artemisinin and structural analogs thereof as inhibitors of the DosS and DosT sensor kinases that activate DosR (Zheng et al., 2017). In this study, transcriptional changes induced by the inhibitors were shown to be similar to transcriptional changes in a DosRST regulon knockout strain and thus support inhibition of the dormancy pathway as the mechanism of action of the inhibitors. This finding, along with the observed inhibition of persistence-associated metabolic processes, such as the synthesis of triacylglycerols, suggests that these inhibitors are able to shift *M. tuberculosis* populations into a metabolically susceptible cell state. Importantly, when concomitantly administered with isoniazid, a first-line antibiotic used to treat *M. tuberculosis* infections, the fraction of cells surviving treatment decreased significantly (Figure 1) (Zheng et al., 2017). Given that artemisinin is already used in clinical settings, there is an opportunity for the rapid translation and adoption of this therapeutic approach.

Treating antibiotic tolerance in *M. tuberculosis* via environmental perturbations of metabolism is also a promising strategy. A growing understanding of the importance of metabolism in inducing antibiotic tolerance in *M. tuberculosis* has enabled the

directed identification of targets for treating tolerant infections (Kim et al., 2013; Nandakumar et al., 2014). For example, this has opened the path for the development of new inhibitors with bactericidal activity that is attributable to the induction of metabolism-linked oxidative shifts within non-replicating *M. tuberculosis* populations (Palde et al., 2016). Furthermore, supplementation of quinolone treatment with glucose and fumarate is effective at treating high-density cultures of *Mycobacterium smegmatis* (Gutierrez et al., 2017). While not yet tested in *M. tuberculosis*, this study provides further support for the development of metabolism-targeted adjuvant therapies.

The development of new drugs and drug combinations that target the dormant state of *M. tuberculosis*, similar to the examples of Pentobra and ADEP4 for *S. aureus*, is an active and exciting area of research. In the case of *M. tuberculosis*, pretomanid and bedaquiline have been shown to be more efficacious against dormant cell states compared to currently established therapies. Pretomanid targets mycolic acid synthesis in aerobic conditions and poisons the respiratory chain by nitric oxide formation, reducing ATP production in anaerobic conditions (Manjunatha et al., 2009; Stover et al., 2000). Bedaquiline also inhibits ATP production, but via direct targeting of the mycobacterial ATP synthase (Andries et al., 2005). Interestingly, despite the ATPase having lower activity in dormant cell states, bedaquiline was found to have increased bactericidal activity (Koul et al., 2008).

The dormancy-targeting properties of these drugs have prompted clinical trials to study drug combinations that incorporate these antibiotics. While proper phase III clinical trials have not yet been completed, the results from one such trial (NC003) indicated that pretomanid and bedaquiline, in combination with pyrazinamide, clear 99% of mycobacteria within 2 weeks in a patient with pulmonary tuberculosis (TBAlliance.org, 2017). Furthermore, the combination of pretomanid and bedaquiline with pyrazinamide is under investigation (NC005) with the aim of reducing treatment duration from 6 months to 3–4 months (TBAlliance.org, 2017). This is especially encouraging in light of the rising rates of antibiotic-resistant *M. tuberculosis* strains for which current regimens have treatment durations of at least 9 to 24 months (Falzon et al., 2017).

Emerging Tools and Approaches for Studying Antibiotic Tolerance

As discussed above, a pathogen's *in vivo* environment plays a fundamental role in inducing antibiotic tolerance and emerging mechanistic insights into such effects are leading to the development of species-specific therapeutic strategies for targeting tolerance. These efforts while impressive, would benefit from improved and more effective means to characterize and study drug-tolerant pathogens in *in vivo* settings. Below, we highlight and describe a select sample of emerging experimental tools and approaches that have the potential to enable researchers and clinicians to better understand the context-specific nature of antibiotic tolerance, as well as design and test improved therapies for treating tolerant infections.

Strategies for Detecting and Quantifying Bacterial Tolerance

The fact that all currently available methods for detecting and quantifying antibiotic tolerance are time and labor intensive directly contributes to the deficit of information available regarding the clinical manifestation of these effects (Brauner et al., 2016). Recent work has focused on the development of assays to detect and quantify the lethal action of bactericidal drugs that are more accessible to clinical microbiology labs than traditional time- and concentration-kill assays (Brauner et al., 2017; Gefen et al., 2017). Traditional disk-diffusion and broth microdilution minimum inhibitory concentration (MIC) assays measure growth inhibition by examining the concentration at which visible bacterial growth is prevented by antibiotic treatment. New assays complement these existing protocols by adding steps for the subsequent removal of antibiotic and addition of replenished nutrients to assess antibiotic lethality as a function of antibiotic concentration and treatment time (Brauner et al., 2017; Gefen et al., 2017). With these assays, it is important to note that the choice of culture conditions will significantly impact the obtained results, and thus attempts to better mimic the infectious environment are needed to generate more clinically relevant information.

The recent discovery of genetic contributors to antibiotic tolerance *in vitro* raises the prospect of using sequence-based technologies to quantify the likelihood that a patient is infected with a strain genetically predisposed to producing a tolerant infection (Brauner et al., 2016; Levin-Reisman et al., 2017; Van den Bergh et al., 2016). In addition to standard whole-genome sequencing, the discovery of tolerance-indicative single-nucleotide polymorphisms (SNPs) suggests that emerging low-cost, point-of-care diagnostic options for SNP detection may be suitable for use in the clinic to inform effective antibacterial therapy (Gootenberg et al., 2017; Köser et al., 2012). Moreover, the identification of RNA signatures of antimicrobial susceptibility linked to resistance suggests that such transcriptional signatures may also be identified and used for the diagnosis of tolerant infections (Barczak et al., 2012).

A current limitation of the above-mentioned approaches is their reliance on the isolation of the pathogen. Unfortunately, this is not always feasible in a clinical setting. Fortunately, recent advances in pathogen biomarker discovery have demonstrated the ability to identify the presence of a specific pathogen using a metabolite signature that can be detected in the serum or breath of infected patients (Pan et al., 2015; Phillips et al., 2012). This raises the possibility that association of specific biomarker profiles with antibiotic-tolerant infections could enable a point-of-care assessment of a patient's infection without requiring isolation of the pathogen.

Approaches for Understanding the In Vivo Context of Infection

Synthetic biology techniques have been utilized to create engineered bacterial sensor strains that can be deployed *in vivo* to record environmental signals (Kotula et al., 2014), as well as detect and report on the replicative state of bacteria exposed to antibiotic treatment (Certain et al., 2017). Similarly, fluorescent reporters have been developed and used to quantify growth dynamics of bacteria isolated from infections (Claudi et al., 2014; Helaine et al., 2014; Manina et al., 2015). Building on this initial progress, new sensor modules could be designed to monitor and characterize additional factors relevant to antibiotic tolerance, including exposure to antibiotics, biofilm formation, and pathogen uptake by host cells.

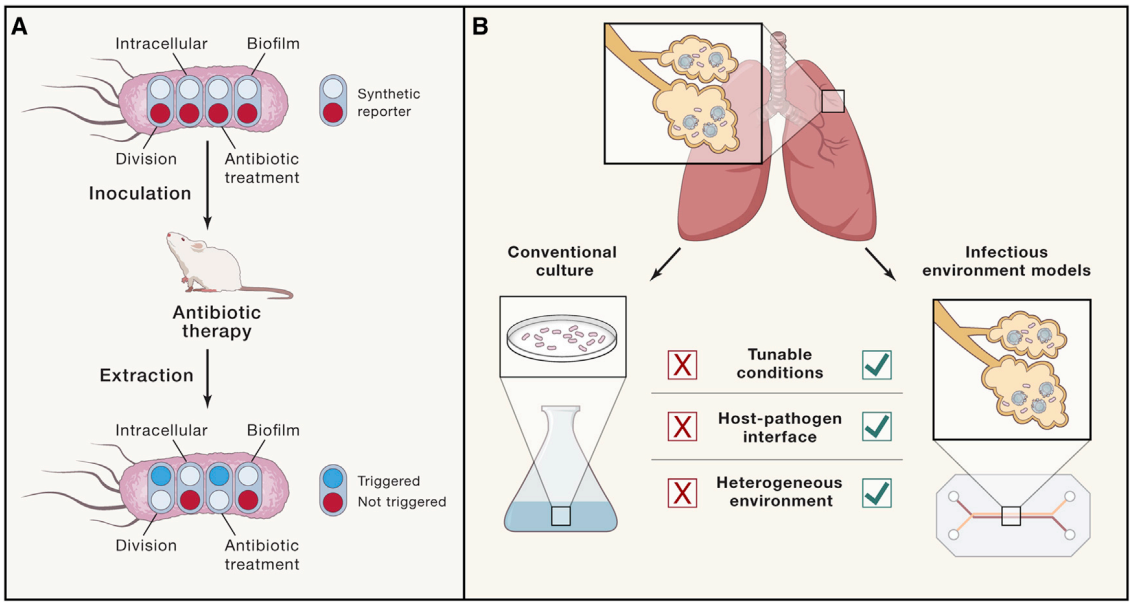

Figure 2. Emerging Tools for Studying the Context-Dependence of Antibiotic Tolerance

The development and adoption of improved *in vivo* and *in vitro* experimental systems are critically needed to study the context-dependent nature of antibiotic efficacy and tolerance.

(A) Engineered bacterial sensor strains enable the characterization of *in vivo* infection dynamics and therapeutic treatment effects. These sensor strains can be customized to detect and report on an array of environmental conditions, bacterial stress responses, and growth behaviors.

(B) Conventional *in vitro* microbial culture systems are over-simplified representations of the clinical context of infection.

Emerging *in vitro* model systems (e.g., organs on chips) aim to more closely replicate the environmental complexities of *in vivo* infection sites.

The development of these synthetic biology technologies raises the possibility of integrating an array of sensors into multiplexed bacterial strains that could be custom-tailored for specific research and clinical applications (Figure 2A). The utilization of these engineered bacterial strains would allow researchers to gain a more comprehensive understanding of the environmental conditions that a bacterial population is exposed to in the host, along with insights into bacterial stress responses triggered *in vivo* by antibiotic treatments. Such tools could provide researchers with the ability to probe key assumptions about *in vivo* population dynamics of bacteria exposed to lethal and sub-lethal concentrations of antibiotics (Certain et al., 2017). These capabilities could also allow researchers to gain a better understanding of the host metabolic environment and how it varies during the course of antibiotic treatment (Yang et al., 2017b), with applications towards manipulating local metabolism to enhance antibiotic efficacy as well as understanding how bacteria emerge from antibiotic-tolerant cell states (Cheverton et al., 2016).

While *in vivo* experiments most closely model the context of an actual patient infection, these can be intractable and rate limiting on several levels, including cost, intervention limitations, and differences between model organisms and human patients. Therefore, recent work has also focused on developing methods for replicating the context of infectious environments outside of a host (Figure 2B). Standard media conditions for cultivating bacteria *in vitro*, for example, are a poor approximation of the host environment. Thus, efforts are being made to better model the environmental conditions of distinct infection sites using different supplemented media conditions (Colmer-Hamood et al., 2016; Farha et al., 2018).

In addition to the nutritional and metabolic environment, it is also desirable to study aspects of the physical architecture of tolerant infections. Recent research in this direction has focused, for example, on the development and characterization of models that more closely mimic the context of a biofilm infection. Recent studies with *S. aureus* and *P. aeruginosa* report on the use of gel-filled glass capillaries and alginate beads, respectively, to approximate *in vivo* biofilm infections (Pabst et al., 2016; Sønderholm et al., 2017), though these methods do not currently include an ability to study the role that host-pathogen interactions play in these infections. In contrast, a new tissue-clearing technique developed for cystic fibrosis sputum allows for the examination of the growth activity and spatial relationship of bacteria and host cells from clinical samples (DePas et al., 2016).

Time-lapse microscopy could also play an important, expanded role in understanding and defining the heterogeneous nature of antibiotic-tolerant phenotypes (Balaban et al., 2004; Wakamoto et al., 2013). Combining time-lapse microscopy with fluorescent reporters for gene expression and ATP biosensors has produced insights into how phenotypic heterogeneity impacts antibiotic efficacy (Maglica et al., 2015; Wakamoto et al., 2013). These studies support the long-standing notion that variation in bacterial metabolism is an important mediator of the variation in antibiotic efficacy that is attributed to phenotypic heterogeneity (Balaban et al., 2004). Time-lapse microscopy combined with the engineering of microfluidic devices has also been used to examine the dynamics of host-pathogen interactions (Delincé et al., 2016), which may prove useful in understanding the role these interactions play in modulating antibiotic tolerance. The expanded development and adoption of

these and related tools should provide a more complete picture of the complex interactions between pathogens and the host environment that lead to antibiotic tolerance and treatment failure.

Concluding Remarks

As highlighted above, antibiotic tolerance plays a critically important, but underappreciated role in the burden of bacterial infections. The manifestation of tolerance varies from pathogen to pathogen, and depends upon each pathogen's physiology and the complexities of their preferred infection sites. However, there are common metabolic processes and cellular signals that govern antibiotic tolerance across many pathogens, enabling the development and implementation of generalizable strategies for targeting tolerance.

The successful translation of these strategies into the clinic will require addressing unique sets of challenges for each therapy. For example, a potential challenge of metabolite-based approaches is the consequence of these environmental modulations on the host. While a metabolite such as mannitol can be achieved at high concentrations in the urinary tract, its osmotic effects might not allow the concentrations necessary to alter the pathogen's physiology in a systemic infection. Similarly, the reported pH changes to potentiate aminoglycosides may be achievable in the urinary tract but not systemically. The response to these implementation challenges must also be shaped by an emerging understanding of the effect of antibiotic treatment on the metabolite profile at infection sites (Yang et al., 2017b). Such limitations may be mitigated by bioengineering strategies, such as site-specific delivery modalities (e.g., engineered liposomes or hydrogels), to concentrate metabolites of interest at the site of infection.

The design and assessment of new treatment strategies relies on an accurate understanding of the clinical manifestation of antibiotic tolerance. However, even with careful experimental control, classic *in vitro* lab techniques fundamentally limit our ability to fully understand how antibiotic tolerance impacts the treatment of clinical infections and its potential contributions to the development of antibiotic resistance. As discussed above, emerging *in vivo* and *in vitro* approaches for studying antibiotic tolerance and infection dynamics should begin to address this gap. Further innovation and development are nonetheless needed to improve such techniques at all scales—from refined single-cell analysis techniques to improved animal models of infection. In particular, we have yet to fully recreate in an *in vitro* model system an infection environment with a host-pathogen interface.

Despite the gaps in our understanding of the complex factors underlying and contributing to antibiotic tolerance, there are still significant reasons for optimism in our battles against tolerant infections. A newfound understanding of the mechanisms responsible for antibiotic lethality and antibiotic tolerance, and how these vary from pathogen to pathogen, has laid the foundation for novel antibiotic tolerance-eradication strategies. It is now the task of researchers in academia and industry to build on this foundation and develop clinically viable therapeutic strategies that can be deployed to reduce the clinical burden of antibiotic tolerance.

ACKNOWLEDGMENTS

We thank Sarah Bening, Bernardo Cervantes, Meagan Hamblin, Allison Lopatkin, Jonathan Stokes, and Jason Yang for critical input and reading of the manuscript. This work was supported by the National Science Foundation Graduate Research Fellowship under award number 1122374, the Defense Threat Reduction Agency under award number HDTRA1-15-1-0051, NIH grant U19AI111276, the Verein zur Förderung von Wissenschaft, Aus-, Weiter- und Fortbildung (VFWAWF-Pool) from the Department of Medicine at the University Hospital of Basel, the Broad Institute Tuberculosis donor group and the Pershing Square Foundation, and a generous gift from Anita and Josh Bekenstein.

DECLARATION OF INTERESTS

S.M. and J.J.C. are co-inventors on patents to the potential adjuvant therapies here discussed. J.J.C. is scientific co-founder and SAB chair of EnBiotix, which is an antibiotics startup company, that has licensed the patents.

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