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Curr Opin Microbiol. Author manuscript; available in PMC 2018 October 16.

Published in final edited form as:

Curr Opin Microbiol. 2017 October ; 39: 73–80. doi:10.1016/j.mib.2017.09.002.

Antibiotic efficacy – context matters

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Abstract

Antibiotic lethality is a complex physiological process, sensitive to external cues. Recent advances using systems approaches have revealed how events downstream of primary target inhibition actively participate in antibiotic death processes. In particular, altered metabolism, translational stress and DNA damage each contribute to antibiotic-induced cell death. Moreover, environmental factors such as oxygen availability, extracellular metabolites, population heterogeneity and multidrug contexts alter antibiotic efficacy by impacting bacterial metabolism and stress responses. Here we review recent studies on antibiotic efficacy and highlight insights gained on the involvement of cellular respiration, redox stress and altered metabolism in antibiotic lethality. We discuss the complexity found in natural environments and highlight knowledge gaps in antibiotic lethality that may be addressed using systems approaches.

Introduction

The discovery of antibiotics early in the 20th century transformed medical practice and microbiological investigation, driving discovery in numerous aspects of microbial physiology including stress responses, mutagenesis and microbial ecology. The primary targets and mechanisms of action for most bactericidal antibiotics have been identified and well-studied [1]; however, there is a growing appreciation that antibiotic lethality is a complex systems-level process that is sensitive to environmental factors [2]. Environmental differences elicit diverse antibiotic treatment responses, from increased susceptibility to phenotypic tolerance. In light of the diminishing pipeline for antibiotic discovery [3], there is an urgent need to better understand mechanisms and factors that influence antibiotic efficacy.

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Conflicts of Interest: J.J.C. is scientific co-founder and SAB chair of EnBiotix, an antibiotics startup company.

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Here, we review recent studies of antibiotic lethality in diverse microbial species. We summarize intracellular mechanisms underlying lethality and describe environmental factors that tune efficacy. We discuss how antibiotics do more than inhibit their primary targets, describe how downstream processes critically participate in active death processes, and highlight insights gained from manipulating extracellular conditions. We also consider the complexity of natural environments and discuss how systems approaches and emerging technologies may provide novel insights into antibiotic lethality.

Bactericidal Processes

Bactericidal antibiotics primarily target processes essential to cellular replication, but studies from several laboratories implicate events downstream of target inhibition as critical components of lethality (Figure 1). This is supported by sequenced strains from experimental evolution studies [4-7] and clinical isolates [8-10] demonstrating that mutations unrelated to an antibiotic target or transport can significantly inhibit antibiotic lethality. In 2007, Kohanski, et al. introduced the hypothesis that bactericidal antibiotics of different classes commonly induce reactive oxygen species (ROS) as part of their lethality [11]. Informed by microarray experiments with *E. coli* cells treated with diverse bactericidal antibiotics, the authors genetically validated ROS-mediated contributions to lethality by tricarboxylic acid (TCA) cycle activity, Fe-S cluster biosynthesis, and SOS-mediated DNA repair. This stimulated further studies revealing the involvement of two-component signaling [12], iron homeostasis [13-15], and nucleotide oxidation [16,17] in ROS-mediated antibiotic lethality; enhanced lethality in cells deficient in oxidative defenses [18]; protection against lethality by mechanisms involving antioxidant defense [19-23]; as well as investigations challenging the general hypothesis [24-26]. These studies have been reviewed and addressed elsewhere [2,27,28].

These investigations and several others demonstrate that antibiotics perturb diverse aspects of bacterial physiology. Such perturbations actively contribute to cell death, in part, by directly inducing cellular damage while also driving the bacterial cell away from homeostasis and inhibiting its ability to cope with antibiotic challenge. Studies on these perturbations have revealed several additional important insights linking aspects of cellular physiology to antibiotic lethality.

Altered Metabolism and Reactive Oxygen Species

Several recent studies have further explored and confirmed the involvement of altered metabolism and ROS in antibiotic lethality using new technologies. Targeted metabolomic studies have revealed significant changes in intracellular energy metabolites following antibiotic treatment [29-31], corroborated by live-cell imaging experiments directly measuring transient changes in ATP [32]. Experiments using the Seahorse XF Analyzer have captured real-time increases to cellular respiration by bactericidal antibiotics [33-35], complementing real-time measurements of overflow ROS production using electrochemical [36] or genetically encoded biosensors [33]. Interestingly, integrated analyses of transcriptomic and metabolomic data have revealed TCA cycle activity to be critical for antibiotic lethality, independent from drug uptake [37]. Collectively, these studies, and

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several others [8,38-45], demonstrate that changes to bacterial metabolism participate in lethality for many bactericidal antibiotics.

Bacteria are naturally optimized for energy efficiency [46,47] and several recent studies suggest metabolic deregulation may also contribute to antibiotic death processes. For instance, penicillin-binding protein disruption by β-lactams stimulates a futile cycle of cell wall synthesis and degradation that depletes cellular resources as part of its toxicity [48,49]. In addition, ATP synthase inhibition by bedaquiline stimulates futile cycling of protons in the respiratory chain [50], which increases oxygen consumption [35] and is lethal to *M. tuberculosis* [51]. Moreover, genetically induced futile cycling in MazF-mediated RNA degradation has been shown to confer complete protection against β-lactam and quinolone lethality, but potentiate sensitivity to aminoglycosides [52]. It is likely that metabolic deregulation by futile cycling constitutes a common mechanism of antibiotic lethality. This is supported by recent evidence that genetically induced futile cycling shares many metabolic features with treatment by bactericidal antibiotics, including increased oxygen consumption, increased ROS production and net decreases in intracellular ATP [53]. Interestingly, cells subjected to genetically induced futile cycling also exhibit increased sensitivity to oxidative stress. Metabolic deregulation may therefore amplify antibiotic-induced stress and enhance lethality.

Translational Stress

Antibiotic lethality is well recognized to decrease under conditions with reduced bacterial growth, and several recent proteomic studies have revealed ribosomal biosynthesis and activity to be key regulators of bacterial growth rate and metabolism [47,54,55]. Macromolecular processes such as protein translation are therefore also likely to be involved in antibiotic lethality. In support of this, bacteriostatic inhibitors of protein translation suppress the lethality of bactericidal antibiotics in wildtype *E. coli* [34], but induce lethality in mutant cells depleted for ribosomal assembly operons [56]. Additionally, bactericidal antibiotics induce several heat shock genes responsive to translational stress [11]. Because growing cells devote >50% of their energy to support the demands of protein translation [57], translational stress likely amplifies the lethal consequences of metabolic stresses induced by antibiotic treatment. Future studies are needed to clarify how antibiotic disruption of translation and other macromolecular processes contributes to lethality.

DNA Damage

Several DNA repair genes are enriched in chemogenomic screens [58,59] and promoter-reporter experiments [60], suggesting DNA damage to also be critical for antibiotic lethality. These findings have been supported genetically, as deletion of SOS response genes enhances drug susceptibility [11,16], while over-expression of mismatch repair genes confers protection [16,33]. Moreover, biochemical inhibition of RecA with polysulfonated compounds potentiates the lethality of multiple antibiotics against both Gram-negative and Gram-positive bacteria [61,62]. Oxidative damage to the nucleotide pool may, in part, underlie this phenotype [29], as incorporation of 8-oxo-guanine induces mismatch repair defects that trigger the formation of double-stranded DNA breaks [16]. Additionally, holliday junction resolvase disruption was recently shown to enhance quinolone lethality in

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mycobacteria [63]. This could be rescued by treatment with bipyridyl, an iron chelator, and thiourea, a hydroxyl radical chelator, supporting prior work implicating antibiotic-induced oxidative stress in damaging DNA [11,16]. Mechanistic studies are needed to further clarify the processes bridging antibiotic target inhibition and lethal DNA damage.

Primary and Secondary Death Processes

Collectively, these studies implicate events downstream of target inhibition as active participants in antibiotic lethality. It is our view that much of the misunderstanding over antibiotic lethality is derived from the difficulty in experimentally separating the lethal contributions of essential gene product inhibition from those of downstream mechanisms. It may be possible to distinguish between these biochemically, as the lethality driven by secondary processes can be interrupted by inhibiting protein translation and cell respiration [34] or by metabolic shunting [37]. Cell death emerging from stress-induced death processes was recently studied in an antibiotic-free system using a historically significant fusion protein [64]. The authors demonstrated that the primary consequence of jamming the SecY protein translocation machinery was cell stasis and that cell death instead emerged from downstream events shared with antibiotic lethality, including ROS accumulation and double-stranded DNA damage. Importantly, nucleotide oxidation occurred hours before cell death in this system. Taken together, these studies support a model for antibiotic lethality where target inhibition drives active death processes by (1) damaging essential cellular processes and (2) inducing stress responses that increase metabolic activity, thereby generating toxic metabolic byproducts, that damage cellular components (Figure 1).

Environmental Factors

Bacterial stress responses evolved to help bacteria adapt to various environments [65] and may confer protection against antibiotic lethality. For instance, pre-treatment with hydrogen peroxide can protect against multiple bactericidal antibiotics by priming oxidative stress responses [33], and nutrient starvation can induce phenotypically tolerant ‘persister’ cells by activating ppGpp and the stringent response [66,67]. Several recent studies demonstrate how environmental factors may alter antibiotic efficacy, providing additional insight into antibiotic lethality (Figure 2).

Oxygen Availability

Antibiotic efficacy decreases in the absence of oxygen [24,25,33] and hyperbaric oxygen can potentiate antibiotic killing of bacterial biofilms [68]. Under normoxia, oxygen participates in aerobic respiration as the terminal electron acceptor for ATP synthesis and produces reactive species as metabolic byproducts [35]. In such conditions, antibiotic efficacy is linked to respiratory activity; for example, deletion of the cytochrome oxidases, which reduces respiration, inhibits drug lethality [34], while deletion of ATP synthase subunits, which increases respiration, enhances lethality [34,69]. Similarly, respiratory suppression by nitrite also inhibits antibiotic killing [70]. Under anoxia, nitrate frequently participates in anaerobic respiration as the terminal electron acceptor and can potentiate antibiotic killing [33]; it is not yet known if nitrate and other terminal electron acceptors also generate toxic metabolic byproducts that contribute to antibiotic death processes. Cellular

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respiration likely affects antibiotic efficacy due to its roles in metabolism and energy production, but may also contribute to lethality by facilitating drug import [37,71] or stimulating intracellular alkalization [72]. Collectively, these studies implicate respiratory activity as an important mediator of the lethal processes induced by antibiotics.

Extracellular Metabolites

Environmental nutrients affect many aspects of bacterial physiology that alter antibiotic efficacy, including growth kinetics and stress response activation [73,74]. Recent studies using flow cytometry and high-throughput assays have revealed how specific carbon metabolites or amino acids may enhance antibiotic death processes by fueling TCA cycle activity, increasing cellular respiration and inducing a proton motive force, promoting both drug uptake and subsequent lethality [37,71,75,76]. Interestingly, flux through the glyoxylate shunt was shown to suppress aminoglycoside potentiation by metabolites such as fumarate despite measureable drug uptake, implicating an active metabolic component to antibiotic lethality [37]. This is supported by recent chemogenetic screens revealing collateral antibiotic sensitivity under nutrient limitation [77]. Additionally, cysteine supplementation was shown to increase cellular respiration, ROS, and antibiotic lethality, as well as inhibit persister formation in *Mycobacterium* [78] through thiol-mediated redox stress [79,80]. To date, input-output relationships between environmental metabolites and antibiotic death phenotypes have not been systematically mapped; such efforts will be important for identifying specific metabolic pathways participating in antibiotic lethality.

Population Heterogeneity

Antibiotic treatment and environmental stresses can give rise to population heterogeneity and confer phenotypic protection against lethality [81]; simple examples include variable expression of multi-drug efflux pumps [82] and stochastic formation of persister cells by toxin-antitoxin systems and the ppGpp-mediated stringent response [83,84]. Recent studies have revealed several additional persister mechanisms, including ATP depletion [85,86], morphological differentiation [87], inter-cell signaling [88,89], and toxin-antitoxin-mediated inhibition of proton-motive force [90,91] or protein translation [92]. Microbial ecologists have largely viewed population heterogeneity as a form of bet-hedging, conserved to facilitate the adaptation to new environments [93]. In support of this, several studies demonstrate how persister mechanisms can help pathogens survive inside host cells [94,95] and provide a reservoir for evolving antibiotic resistance [96-98]. Investigations have also shown how social behaviors such as quorum sensing [99], signaling [100] and cooperative mutualism [101] can confer protection against antibiotic lethality in mixed-species environments. Nascent studies on population-level responses to antibiotic treatment have revealed several intra- and inter-species mechanisms for collective protection [102,103], but such experiments are challenging due to scale. Advances in synthetic biology and microfluidic technologies are now poised to enable significant insight into ecological mechanisms participating in antibiotic efficacy.

Multidrug Contexts

Antibiotic treatment outcomes are diverse in multidrug environments, and previous exposure to an antibiotic can alter the efficacy of other antibiotics [104]. Studies on multidrug

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contexts highlight the roles of cellular respiration [34], ATP synthesis and polysaccharide synthesis [69] in determining treatment efficacy. Pretreatment with sub-lethal doses of single antibiotics induces physiological stress responses that protect against antibiotic lethality [60] and other environmental stresses [105]. Extended antibiotic exposure leads to genetically encoded resistance with collateral sensitivity or resistance to antibiotics of different classes [106], which may be exploited to potentiate population-level lethality by antibiotic cycling [107,108]. Recent efforts integrating metabolomic profiling with experimental evolution have revealed how bacterial metabolism may constrain the acquisition of antibiotic resistance and differential cross-resistance [109]. Mechanistic details explaining antibiotic synergy and antagonism remain nascent; future studies on multidrug efficacy and resistance would benefit from integrated exploration of environmental and physiological factors.

Perspectives

Although antibiotics are routinely studied under well-controlled lab conditions, the natural environments in which antibiotics are typically found and used are complex in nutrient availability, microbial heterogeneity, and other external stressors. Because environmental factors can elicit diverse effects on antibiotic lethality, careful consideration must be paid to experimental conditions to minimize confounding effects by contextual elements. Several open questions remain with respect to antibiotic lethality in natural contexts, including antibiotic-induced changes to the extracellular environment and the dynamic interactions between different species sharing a common ecological niche. Moreover, understanding the differences in microbial physiology between host metabolic environments and the nutrient rich conditions commonly used to study bacterial pathogens will be important for translating *in vitro* discoveries into clinical care. Further understanding on how external effectors act on bactericidal processes will require systems approaches to meet the challenges posed by such complexity.

Antibiotic lethality is increasingly understood to be driven by active death processes resulting from both target inhibition and subsequent induction of stress responses. Compensatory changes to bacterial physiology likely participate bi-directionally, furthering downstream cellular damage and modulating the direct upstream consequences of target inhibition. For instance, alterations in cellular metabolism or protein translation may have far-reaching consequences on target availability and the biochemistry of drug-target interactions. Future studies are needed to clarify the relative contributions of primary and secondary death processes.

Recent advances in experimental technologies and quantitative modeling are now poised to enable additional systems-level insights into antibiotic lethality. Mass spectrometry allows interrogation into spatial determinants of antibiotic efficacy [110,111] and synthetic biology has contributed novel tools for perturbing essential genes [112]. Current quantitative models are providing mechanistic insights into several nonlinear components of antibiotic lethality [56,113] and are useful for predicting multidrug treatment outcomes [114,115] and antibiotic drug synergies [116,117]. Integration of these tools will enable identification of additional mechanistic details between primary target inhibition and subsequent lethality.

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Conclusion

Antibiotics naturally evolved as inhibitory agents for microbial warfare and have provided insights into mechanisms underlying bacterial cell death. Recent studies have revealed that several events downstream of primary target inhibition actively contribute to antibiotic lethality, including alterations to cellular metabolism, protein translation, and DNA damage. These processes are sensitive to environmental cues and future studies will be needed to better understand antibiotic efficacy in natural settings. Systems approaches have the potential to accelerate such efforts and provide additional mechanistic insight into antibiotic lethality.

Acknowledgments

This work was supported by the National Institutes of Health under award numbers K99GM118907 and U19AI111276, the National Science Foundation Graduate Research Fellowship under award number 1122374, the Defense Threat Reduction Agency under award number HDTRA1-15-1-0051, and a generous gift from Anita and Josh Bekenstein.

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Highlights

- Antibiotic lethality involves processes downstream of primary target inhibition.
- Respiration and altered metabolism actively participate in antibiotic lethality.
- Environmental cues alter antibiotic efficacy via metabolism and stress responses.
- Systems approaches are important for studying antibiotics in complex environments.

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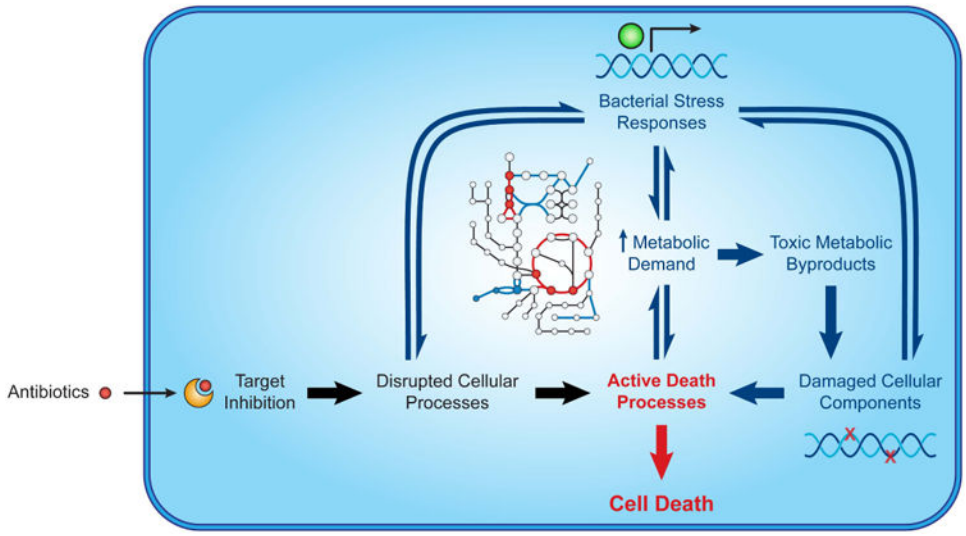

Figure 1.

Antibiotics induce active death processes underlying lethality. Target inhibition directly triggers lethality by disrupting essential cellular processes (black). Stress responses induced by such disruptions indirectly trigger lethality by increasing metabolic demand and generating metabolic byproducts that damage cellular components (e.g., DNA, proteins, lipids) (blue). Environmental factors tune antibiotic lethality by acting on stress responses and/or altering bacterial metabolism.

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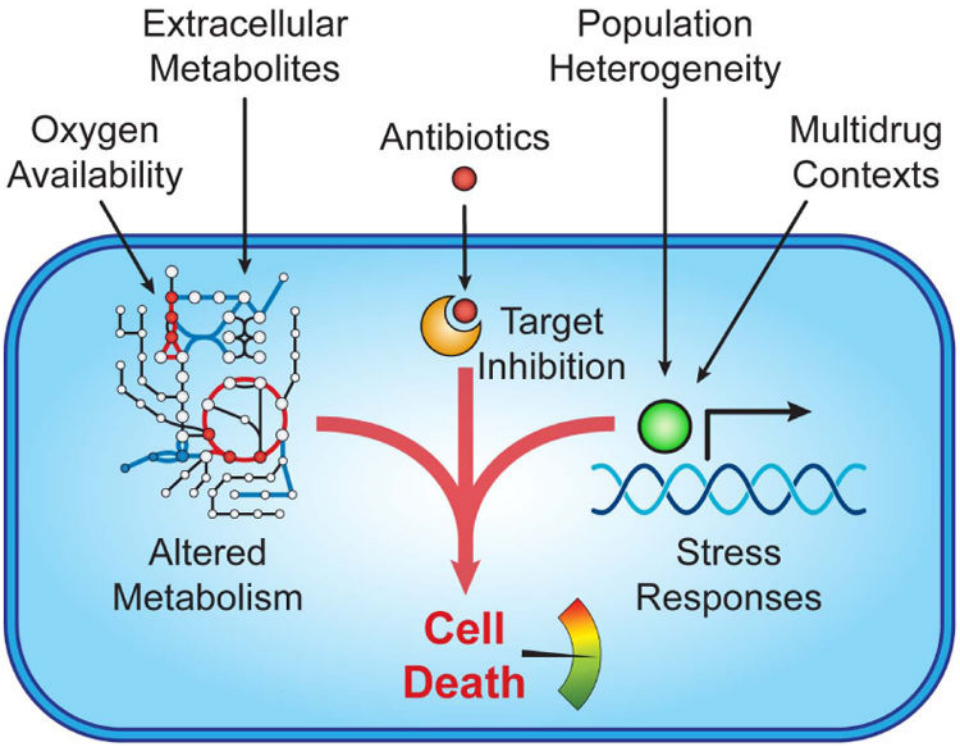

Figure 2.

Environmental factors tune antibiotic lethality. Cues such as oxygen availability and extracellular metabolites impact cell death by acting on cell metabolism. Population heterogeneity and multidrug contexts protect against lethality by inducing stress responses and defense mechanisms.