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From the soil to the clinic: The impact of microbial secondary metabolites on antibiotic tolerance and resistance

Elena K. Perry[1,*], **Lucas A. Meirelles**[1,*], **Dianne K. Newman**[1,2,+]

[1]Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, California, USA

[2]Division of Geological and Planetary Sciences, California Institute of Technology, Pasadena, California, USA

Abstract

Secondary metabolites profoundly affect microbial physiology, metabolism and stress responses. Increasing evidence suggests that these molecules can modulate microbial susceptibility to commonly used antibiotics; however, secondary metabolites are typically excluded from standard antimicrobial susceptibility assays. This may in part account for why infections by diverse opportunistic bacteria that produce secondary metabolites often exhibit discrepancies between clinical antimicrobial susceptibility testing results and clinical treatment outcomes. In this Review, we explore which types of secondary metabolites alter antimicrobial susceptibility, as well as how and why this phenomenon occurs. We discuss examples of molecules that opportunistic and enteric pathogens either generate themselves or are exposed to from their neighbors, and the nuanced impacts these molecules can have on tolerance and resistance to certain antibiotics.

Graphical Abstract

In this Review, Perry, Meirelles and Newman review the growing body of evidence that microbial secondary metabolites can modulate susceptibility to commonly used antibiotics, focusing on the mechanisms and why this phenomenon occurs, and they discuss the implications for the diagnosis of antibiotic resistance and therapeutic strategies.

Introduction

A vast number of organisms, many of which hail from the soil, produce a wide range of molecules classified as ‘secondary metabolites’[1,2]. They can be generated by Eukarya (for example, plants and fungi), Bacteria and Archaea, and are usually defined as organic compounds that do not directly support the producer’s growth or development[3–5]. In microbial planktonic cultures, they are typically produced during stationary phase, once

+ dkn@caltech.edu .

*These authors contributed equally.

Author contributions

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doubling times have slowed[6,7]. As a result, these compounds were for many years assumed to be waste products of metabolism[8]. However, a more nuanced view of the biological functions of microbial secondary metabolites has emerged over the past two decades. Indeed, the moniker ‘secondary’ is something of a misnomer, as these molecules have been shown to have key roles in multiple physiological processes that are critical for microbial survival[6,7], including but not limited to the acquisition of nutrients (such as iron or phosphate), cell–cell signaling and energy conservation in the absence of oxygen[2,9–12].

In addition to conferring such pleiotropic benefits, many microbial secondary metabolites are also toxic, both to their producers and to neighboring organisms[1,2,8]. It is therefore not surprising that antibiotic development pipelines have driven the majority of secondary metabolite characterization and purification efforts, dating back to the discoveries of penicillin and streptomycin in the early 20th century. Most modern clinical antibiotics are derivatives of natural products that originated from soil microorganisms, and the soil-to-clinic axis continues to inspire natural product chemists in their search for and design of new drugs[13]. Yet, microbiologists have neglected to consider the potential for unintended consequences of this pipeline, particularly with respect to antibiotic efficacy against opportunistic pathogens that evolved in the same environment. Microorganisms are rarely, if ever, found in isolation, and therefore the presence of secondary metabolites in a microbial community exerts evolutionary pressure both on secondary metabolite-producing and non-producing members to develop means to withstand them. These defenses can in turn have collateral activity against clinical antibiotics.

In this Review, we highlight the growing body of evidence connecting bacterial secondary metabolites to the phenomena of antibiotic tolerance [G] (that is, the ability to survive transient antibiotic exposure) and resistance [G] (that is, the ability to grow in the presence of antibiotics at a given concentration)[14–16]. We also use the more generic term ‘antibiotic resilience’ [G] to refer to the ability of a bacterial population to be refractory to antibiotic treatment, which can arise from an increase in tolerance and/or resistance. Emphasizing examples of secondary metabolites produced by opportunistic or enteric pathogens (Table 1), we discuss common modes of action through which these molecules can alter antibiotic efficacy in both single-species and polymicrobial communities. Specifically, we draw attention to secondary metabolites that regulate multidrug resistance efflux systems, secondary metabolites that modulate the toxicity of antibiotics through interactions with reactive oxygen species, and the potential for secondary metabolite-induced antibiotic tolerance to provide an overlooked route for the evolution of antibiotic resistance. Although the impact of bacterial secondary metabolites on infection treatment outcomes has yet to be addressed in clinical studies, *in vitro* data indicating changes in antimicrobial susceptibility in the presence of diverse secondary metabolites strongly suggest that these molecules may represent an underappreciated factor in the recalcitrance of many opportunistic and chronic infections. We offer recommendations for future experiments to explore the breadth of relevance of these observations (Box 1) and discuss the implications that secondary metabolite production can have for the diagnosis of antibiotic resistance (Box 2). Finally, we consider how knowledge of interactions between secondary metabolites and antibiotic efficacy could be applied to optimize the use of existing antimicrobial drugs and generate targets for novel therapeutic strategies.

Induction of efflux systems

Activation of efflux pumps [G] that export toxins out of the cell is one mechanism used by diverse bacteria to thrive during clinical antibiotic treatment[17,18]. However, efflux pumps long predate human use of synthetic antibiotics, and therefore are presumed to have originally evolved to transport other, naturally occurring substrates, such as secondary metabolites[19,20]. The types and components of efflux pumps have been extensively reviewed[21,22]. In this Review, we focus on how the induction of efflux systems in response to self-produced secondary metabolites can affect antibiotic tolerance and resistance in pathogenic bacteria (Fig. 1A). The examples we discuss fall mostly within the resistance-nodulation-division (RND) efflux systems, but the same principles could, in theory, be applicable to other types of efflux systems that are regulated by secondary metabolites. Importantly, efflux pumps vary in their specificity, with regard to both their regulation and their substrate affinity[22]. Therefore, to predict whether a secondary metabolite will increase antibiotic resilience in its producer through the induction of a particular efflux system, it is essential to understand how the secondary metabolite interacts with the transcriptional regulation of the system, as well as which classes of drugs the system can transport. Many known efflux-regulating secondary metabolites have at least one aromatic or heterocyclic ring (Fig. 1B), possibly suggesting that secondary metabolites with this structural motif are particularly likely to affect antibiotic resilience through the induction of multidrug efflux systems.

In the enteric bacterium *Escherichia coli*, one of the best-studied multidrug resistance efflux systems is AcrAB–TolC, which has a complex regulatory system and an extensive substrate range, being part of a general stress response[22,23]. Although AcrAB–TolC is generally expressed at high intrinsic levels[22,24], numerous molecules have been shown to further upregulate its transcription, including self-produced secondary metabolites such as the compound 2,3-dihydroxybenzoate, an intermediate in the biosynthesis of the siderophore enterobactin[25]. In fact, 2,3-dihydroxybenzoate directly binds to MarR[26], a transcriptional repressor that modulates the expression of AcrAB–-TolC alongside the redox-sensing SoxRS regulatory system[22] (Fig. 1C). Although it has not been directly tested whether the production of enterobactin or 2,3-dihydroxybenzoate *per se* increases antibiotic resilience, it is well established that AcrAB–TolC provides protection against many classes of clinical antibiotics[23]. The signaling molecule indole is another example of a self-produced secondary metabolite in *E. coli* with an efflux-mediated effect on antibiotic susceptibility. Indole triggers the expression of certain multidrug efflux pumps in enteric bacteria[27–30]. Indeed, the production of high levels of indole by a subpopulation of mutants has been characterized as a ‘charity’ mechanism that induces population-level resistance against norfloxacin and gentamicin in *E. coli*, with the MdtEF–TolC efflux system being upregulated by this secondary metabolite[31]. Intriguingly, a *tolC* mutant of *Shewanella oneidensis*, an environmental isolate related to opportunistic pathogens of fish[32], was sensitive to anthraquinone-2,6-disulfonate (an analog of naturally occurring redox-active humic substances in soils and sediments that resembles quinone-containing synthetic antibiotics)[33], further suggesting that functional relationships between structurally similar natural and synthetic molecules may be common.

Efflux pumps can also provide protection against secondary metabolites that are toxic to their producers, an effect that might confer resilience to clinical antibiotics. For example, in the opportunistic pathogen *Pseudomonas aeruginosa*, the redox-sensing transcription factor SoxR (Fig. 1C) activates expression of the MexGHI–OpmD efflux system in response to phenazines, which are toxic, redox-active self-produced secondary metabolites[9,34–36]. Phenazines have important roles both in natural environments (for example, by protecting plants against fungal pathogens) as well as in infections (for example, by increasing *P. aeruginosa* virulence in the lungs of patients with cystic fibrosis)[37,38]. The phenazine pyocyanin (PYO) also induces a second efflux operon in *P. aeruginosa*, *mexEF–oprN*[35,36], which is clinically relevant[39,40]. Experiments with a broad-spectrum efflux pump inhibitor that reduces the activity of various RND efflux systems[41,42], as well as a knockout mutant lacking the *mexGHI–opmD* operon, have confirmed that efflux is a major mechanism underlying the tolerance and resistance of *P. aeruginosa* to its own phenazines[34,36,43]. Importantly, both phenazine-regulated efflux pumps are also known to transport fluoroquinolones, and multiple studies have reported a strong antagonistic effect of phenazines on fluoroquinolone efficacy[36,43–45]. For example, *P. aeruginosa* cells exposed to PYO (either self-produced or exogenously added) display increased tolerance against the fluoroquinolones ciprofloxacin and levofloxacin[36]. This phenotype was recapitulated in one study by artificially overexpressing MexGHI–OpmD in a phenazine-null mutant to a level similar to that achieved in the presence of PYO, which suggests that drug efflux drives PYO-mediated increases in fluoroquinolone tolerance[36]. Interestingly, besides fluoroquinolones and chloramphenicol, MexEF–OprN is also thought to transport trimethoprim and sulfamethoxazole, to which *P. aeruginosa* is intrinsically resistant[46], but not aminoglycosides[46], against which phenazines have demonstrated mixed effects on tolerance[36,44,45]. Like phenazines, but unlike aminoglycosides, all of the known antibiotic substrates for MexEF–OprN have at least one aromatic ring (Fig. 1B), which suggests that analysis of shared structural motifs may enable prediction of which clinical antibiotics will be most affected. Such structural comparisons would only be appropriate for efflux systems that are relatively specific in substrate recognition, and would not apply to efflux systems that export a wide range of antibiotics[46].

In addition to MexGHI–OpmD and MexEF–OprN, *P. aeruginosa* possesses at least nine other efflux systems that belong to the RND family, many of which have been studied in detail with respect to their structures and biochemistry[22,46–48]. Whether any *P. aeruginosa*-produced secondary metabolites regulate these other efflux systems under clinically relevant circumstances remains to be determined. Notably, however, phenazines are not the only secondary metabolites produced by *P. aeruginosa* that promote increased resilience against antibiotics by inducing efflux. Recent work showed that production of the secondary metabolite paerucumarin also stimulates transcription of the MexEF–OprN efflux system in *P. aeruginosa*, with consequent increases in resistance to both chloramphenicol and ciprofloxacin[49,50]. These findings underscore the potential for self-produced secondary metabolites to promote resilience to clinical antibiotics in opportunistic pathogens by triggering the upregulation of efflux pumps.

Other bacterial opportunistic pathogens inhabiting soils or plant roots also produce secondary metabolites that promote efflux and decrease antibiotic susceptibility. For

example, several strains of '*Burkholderia cepacia* complex' species isolated from patients with cystic fibrosis can produce salicylate[51,52]. Salicylate has Fe-chelating properties and is used as a siderophore by the producing cells[53,54]. It also induces specific efflux systems in *Burkholderia* species (for example, CeoAB–OpcM in *B. cenocepacia*), which leads to increased antibiotic resistance[55]. The salicylate-derived antibiotic resistance effect is not limited to the *Burkholderia* genus; for example, in enterobacteria, salicylate binds to and inactivates MarR, which leads to upregulation of efflux pump expression and increased resistance to multiple clinical antibiotics[56,57]. *Burkholderia* species also produce several other secondary metabolites that could affect antibiotic resilience, including many natural antibiotics with strong inhibitory capacities relevant during plant host colonization[58–61]. One intriguing example is toxoflavin, which is produced by several *Burkholderia* species, including *Burkholderia gladioli*, a common species found in patients with cystic fibrosis[62,63]. Although it is still unknown whether toxoflavin poisons producing cells, it is redox-active[64–66], presumably causing oxidative stress through the generation of H_2O_2 (Ref. [65]), and it is toxic to other bacteria and fungi[65,67]. More importantly, like PYO, toxoflavin induces a specific RND efflux system, ToxFGHI, which is used for its export[68]. It is not yet known if *B. gladioli* or other opportunistic pathogens within the *Burkholderia* genus produce toxoflavin during infections, or if the toxoflavin-induced RND efflux system ToxFGHI can transport any of the currently used clinical antibiotics; however, this is a possibility, given that toxoflavin, like PYO, bears structural similarity to fluoroquinolones.

Finally, we note that the above examples of efflux system induction due to the presence of secondary metabolites are all from Gram-negative bacteria, which are traditionally the organisms in which drug efflux has been studied in more detail. However, a related phenomenon has been demonstrated in the non-pathogenic Gram-positive bacterium *Streptomyces coelicolor*, which produces a natural antibiotic, actinorhodin, that stimulates expression of a transporter similar to those that export tetracycline[69,70]. Future work should investigate the extent to which secondary metabolite-mediated induction of multidrug efflux pumps might occur in pathogenic Gram-positive bacteria and its consequences for antibiotic resilience. Taken together, the examples discussed highlight the rich diversity of bacterial secondary metabolites that induce efflux activity in their producers, potentially compromising the efficacy of clinical antibiotics.

Modulation of oxidative stress

More than a decade ago, it was proposed that bactericidal antibiotics exert their lethal effects in part by inducing oxidative stress, regardless of the specific cellular targets of different antibiotic classes[71]. Although this hypothesis has engendered controversy[72,73], evidence reviewed elsewhere[74] suggests that bactericidal antibiotics have an impact on cellular redox states, and that the resulting increases in reactive oxygen species (ROS) and oxidative stress can contribute to cell death. Importantly, many secondary metabolites also interface with cellular redox homeostasis and oxidative stress responses. In this section, we discuss three different modes of action by which these metabolites can potentially antagonize or potentiate the toxicity of clinical antibiotics (Fig. 2A): upregulation of oxidative stress response genes; direct detoxification of ROS; and increased endogenous ROS generation.

Upregulation of defenses against oxidative stress.

Secondary metabolites that upregulate the expression of oxidative stress responses can prime bacterial cells for tolerance and/or resistance to clinical antibiotics, analogous to the protective effects of exposure to sub-lethal concentrations of oxidants like H_2O_2. Among this class of metabolites, indole is perhaps the best-studied. As mentioned above, indole can also influence antibiotic susceptibility by upregulating efflux pump expression. However, this effect is thought to happen primarily at high concentrations of indole (>1 mM)[27,29]; indole concentrations in human feces tend to be lower[75], though measurements up to the equivalent of 1100 μM have been recorded[76]. At these lower concentrations, indole is non-toxic to its producer, *E. coli*, but still induces oxidative stress response genes regulated by OxyR, including alkyl hydroperoxide reductases, thioredoxin reductase, and the DNA-binding protein Dps[77]. Exposure to indole increases the frequency of *E. coli* persisters [G] to antibiotics that belong to three different classes (fluoroquinolones, aminoglycosides and β-lactams) by at least an order of magnitude, and deletion of *oxyR* substantially diminishes this effect, which demonstrates that upregulation of oxidative stress responses by a secondary metabolite can contribute to bacterial persistence[77]. The molecular pathway through which indole activates OxyR remains unclear, but indole readily undergoes one-electron reduction to a radical form in the presence of hydroxyl radicals or other strong oxidants[78], which suggests that it might interact with and potentially amplify endogenous ROS generated as a byproduct of respiration. Indole has also been proposed to disrupt the arrangement of membrane lipids, which would enable the direct interaction of respiratory quinones with dioxygen and thereby lead to the generation of superoxide[79].

PYO is another example of a bacterial secondary metabolite that induces oxidative stress responses. As a redox-active metabolite that can gain and lose electrons reversibly under physiological conditions (Fig. 2B), PYO can generate ROS under aerobic conditions through direct reduction of oxygen to superoxide (Fig. 2C), in addition to interfering with respiration[80,81]. In its producer, *P. aeruginosa*, PYO increases superoxide dismutase activity[82] and upregulates the transcription of several other oxidative stress response genes, including those encoding alkyl hydroperoxide reductases, thioredoxin reductase, catalase and iron-sulfur cluster biogenesis machinery[35] (Fig. 2C–D). Intriguingly, PYO has been shown to increase the frequency of gentamicin-resistant mutants in *P. aeruginosa* cultures in a manner that is independent of drug efflux, as PYO does not upregulate aminoglycoside-transporting efflux pumps[36]. Given that gentamicin is known to promote increased intracellular ROS levels through the formation of complexes with iron[83,84], and that pre-treating cells with oxidants can prime them to tolerate antibiotics[85], a plausible explanation for this phenomenon is that PYO-induced oxidative stress responses counteract ROS-related gentamicin toxicity. This in turn could decrease the rate at which spontaneous mutants are stochastically lost from the population[86], which would lead to the observed increase in the frequency of resistant mutants. PYO can also promote the growth of *P. aeruginosa* in the presence of other aminoglycosides (kanamycin, streptomycin and tobramycin) and a β-lactam antibiotic (carbenicillin)[45]. Like gentamicin, these antibiotics are not known to be substrates for PYO-regulated efflux systems[36,46], but they belong to classes of drugs that have been shown to perturb cellular redox states[87,88], which again

suggests that the observed decreases in antibiotic efficacy could be related to PYO-induced oxidative stress responses.

Detoxification of ROS.

In contrast to ROS-generating redox-active secondary metabolites that induce enzymatic oxidative stress responses, secondary metabolites that possess antioxidant activity [G] can protect against antibiotic assaults by directly detoxifying antibiotic-derived ROS. One example is ergothioneine, which is one of two major sulfur-containing redox buffers in mycobacteria, along with mycothiol. Loss of ergothioneine biosynthesis genes in *Mycobacterium tuberculosis* decreases minimum inhibitory concentrations (MICs) for rifampicin, isoniazid, bedaquiline and clofazimine, in addition to decreasing survival under treatment at the wildtype MICs by at least 30–60% (Ref. [89]). Other secondary metabolites with antioxidant activity have been shown to be important for resistance to ROS generated by host immune cells, including macrophages and neutrophils. One such metabolite is staphyloxanthin, a membrane-embedded carotenoid pigment that protects *Staphylococcus aureus* against ROS[90,91] and consequently decreases killing by neutrophils[91]. Likewise, carotenoids produced by group B *Streptococcus*[92] and the dental pathogen *Streptococcus mutans*[93] have been implicated in resistance to ROS. The antioxidant capacity of these pigments has been attributed to their highly conjugated polyene backbones[94], though the exact mechanisms by which different carotenoids scavenge ROS remain unclear[95]. Importantly, antioxidant agents need not necessarily be located in the cytoplasm to protect against antibiotic-induced ROS accumulation, as exogenous catalase has been shown to increase bacterial survival following exposure to trimethoprim[96]. Thus, although most studies on membrane-embedded carotenoids have focused on interactions with extracellular sources of ROS, these pigments might also be able to dampen oxidative stress that originates inside the cell during treatment with bactericidal antibiotics. Considering the growing body of evidence that redox imbalance and oxidative stress are downstream effects of many antimicrobial drugs[74], the possibility that staphyloxanthin or other membrane-associated pigments promote resilience to clinical antibiotics is worthy of further investigation.

In addition to the above examples, certain microbially produced compounds that are not classic secondary metabolites, either because they are inorganic or are not always dispensable for normal growth, also contribute to antibiotic tolerance or resistance by enhancing the antioxidant capacity of bacterial cells. For example, endogenously generated H_2S protects a diverse range of bacteria, including *E. coli*, *P. aeruginosa* and *S. aureus*, against the toxicity of antibiotics known to exert oxidative stress, such as gentamicin[97]. This phenomenon has been proposed to stem from a dual-action mechanism whereby H_2S both inhibits the Fenton reaction and stimulates the activities of catalase and superoxide dismutase[97]. Polyamines have also been shown to protect bacteria from antibiotic toxicity by counteracting oxidative stress. This is thought to be due in part to their capacity to neutralize free radicals[98,99], though physical protection of cellular components and indirect upregulation of other oxidative stress responses may also be involved[98,100]. *E. coli* upregulates production of putrescine and spermidine upon exposure to antibiotics under aerobic conditions, and these polyamines in turn statistically significantly increase viability under antibiotic treatment by decreasing ROS production and oxidative damage[101].

Similarly, putrescine secreted by *Burkholderia cenocepacia* protects its producer against oxidative stress arising from treatment with polymyxin B, norfloxacin, or rifampicin[99]. Finally, in *P. aeruginosa*, the BqsRS regulatory system drives increased polyamine production upon sensing ferrous iron, which is prevalent in the lungs of patients with cystic fibrosis, thereby promoting survival in the presence of cationic antibiotics such as polymyxins and aminoglycosides[102]. Together, these examples demonstrate how interactions between diverse bacterial metabolites and oxidative stress can lead to increased resilience against clinical antibiotics.

Synergistic interactions between secondary metabolites and antibiotics.

Besides the examples in which secondary metabolites decrease antibiotic efficacy by attenuating oxidative stress, it is also important to note that in some cases, secondary metabolites that increase ROS generation can amplify the toxicity of clinical antibiotics. One example is 2-heptyl-3-hydroxy-4-quinolone, also known as the *Pseudomonas* quinolone signal (PQS), which is produced by *P. aeruginosa*. PQS is a redox-active molecule that can reduce not only free radicals but also metal ions, and consequently possesses both antioxidant properties and pro-oxidant activity [G], as reduction of iron promotes ROS formation through the Fenton reaction[103]. The pro-oxidant activity seems to dominate in cells, as PQS induces oxidative stress responses and increases sensitivity to hydrogen peroxide and ciprofloxacin[103,104]. The pro-oxidant activity of PQS is also evidenced by the fact that overproduction of PQS acts synergistically with impairment of superoxide dismutase and catalase activity to increase endogenous oxidative stress and antibiotic susceptibility[105]. Notably, abolishment of PQS production increases tolerance to ciprofloxacin, imipenem and gentamicin[103]. Another redox-active secondary metabolite that increases antibiotic susceptibility under certain conditions is PYO. Although pre-exposure of *P. aeruginosa* to PYO increases tolerance to fluoroquinolones and promotes the establishment of gentamicin-resistant mutants, PYO and other phenazines have also been shown to increase the sensitivity of *P. aeruginosa* to cationic antimicrobial peptides, including colistin[36,44] and polymyxin B[45]. The underlying mechanism of this synergistic interaction has yet to be determined, but it is notable that polymyxin B precipitates severe oxidative stress in *P. aeruginosa* and other Gram-negative opportunistic pathogens[106,107]. Moreover, unlike fluoroquinolones or aminoglycosides, cationic antimicrobial peptides also permeabilize the outer membrane[108] and consequently might increase phenazine uptake, which would accelerate ROS generation even further. Thus, the synergy between phenazines and cationic antimicrobial peptides may ultimately be driven by an overwhelming cascade of oxidative stress. Given that ROS-generating secondary metabolites can both potentiate and diminish antibiotic efficacy depending on the circumstances, future studies focused on revealing which of these effects take precedence during infections will be critical to better understand how such secondary metabolites may affect clinical treatment outcomes.

Interspecies antibiotic resilience

So far, we have discussed examples of secondary metabolites that either are known to affect their producer's susceptibility to clinical antibiotics, or have the potential to do so based on their interactions with established mechanisms of antibiotic tolerance and resistance.

However, equally important is the fact that many secondary metabolites, in particular those that are secreted, can also modulate interspecies antibiotic resilience (Fig. 3). Indeed, how interactions among members of a polymicrobial infection [G] might affect antibiotic treatment outcomes has recently received much attention[109,110].

Although our understanding of how exchangeable secondary metabolites affect community-level antibiotic resilience is still in its infancy, there have been several reports on this subject. For example, indole has been identified as an interspecies modulator of antibiotic tolerance between *E. coli* and *Salmonella enterica* subsp. *enterica* serovar Typhimurium[29,111]. *S.* Typhimurium, which is likely to interact with commensal *E. coli* during infection, does not produce indole, yet its tolerance to ciprofloxacin increased by greater than threefold in the presence of exogenously added indole, as well as in co-cultures with indole-producing *E. coli*[111]. Similar to its effect in *E. coli*, indole induces OxyR-regulated oxidative stress responses in *S.* Typhimurium, and deletion of *oxyR* abolished the indole-mediated increase in ciprofloxacin tolerance[111]. Interestingly, indole has also been reported to increase resistance to ampicillin in the indole non-producer *P. aeruginosa*, not by inducing oxidative stress responses but rather by stimulating the expression of efflux pumps and a chromosomal β-lactamase[112]. These examples highlight the potential for secreted secondary metabolites to have both conserved and mechanistically divergent effects on antibiotic resilience in neighboring species.

Other secondary metabolites besides indole have shown potential as interspecies modulators of antibiotic resilience. For example, the protective effect against polymyxin B of putrescine secreted by *B. cenocepacia* extends not only to the producer, but also to neighboring species in co-cultures, including *E. coli* and *P. aeruginosa*[100]. Similarly, PYO produced by *P. aeruginosa* considerably increases the tolerance of multiple clinically relevant *Burkholderia* species to ciprofloxacin in co-cultures[36]. In yet another opportunistic pathogen, *Acinetobacter baumannii*, exposure to PYO increases the frequency of persisters to amikacin and carbenicillin by three- to four-fold[113], possibly through the upregulation of superoxide dismutase and catalase[113,114]. Finally, quorum sensing signals produced by *P. aeruginosa* induce fluconazole resistance in the yeast *Candida albicans*[115], which indicates that bacterial secondary metabolites can mediate not only interspecies but also interkingdom effects on antimicrobial efficacy. So far, these interactions have only been demonstrated *in vitro*. However, given that *Burkholderia* species, *Acinetobacter* species and fungal pathogens can all be found together with *P. aeruginosa* in chronic infections[62,116,117], these findings suggest that valuable insights could be gained from future studies focused on the *in vivo* relevance of secondary metabolite-mediated interspecies induction of antibiotic resilience.

Importantly, although the above examples suggest that certain secreted secondary metabolites have the potential to raise the community-wide level of antibiotic resilience in polymicrobial infections, a secondary metabolite that promotes antibiotic resilience in one species will not always do so in others. One key consideration is that in order for a secondary metabolite to trigger cross-species induction of resilience to clinical antibiotics, the non-producing species must be able to tolerate any stress caused by the secondary metabolite. If toxicity outweighs benefits from defense induction or antibiotic detoxification, the non-producing species would not gain a benefit. In fact, the secondary metabolite might

even act synergistically with the clinical antibiotic[118]. Examples of this type of interaction have been found between *S. aureus* and *P. aeruginosa*, two species that often co-occur in patients with cystic fibrosis[119–121]. *S. aureus* is sensitive to several secondary metabolites secreted by *P. aeruginosa*[122,123], and some of these can increase the susceptibility of *S. aureus* to clinical antibiotics. For example, *P. aeruginosa*-produced rhamnolipids potentiate tobramycin toxicity in *S. aureus* by increasing membrane permeability[122]. Another *P. aeruginosa*-produced secondary metabolite, 2-n-heptyl-4-hydroxyquinoline N oxide (HQNO), was recently shown to increase the sensitivity of *S. aureus* biofilms to fluoroquinolones and membrane-targeting antibiotics via a similar mechanism[124]. However, the toxic effects of *P. aeruginosa* secondary metabolites on *S. aureus* are not always synergistic with clinical antibiotics. By inhibiting growth, HQNO promotes tolerance in *S. aureus* biofilms specifically to antibiotics targeting cell wall synthesis and protein synthesis[125], contrary to its effect on other classes of antibiotics. By contrast, in planktonic cultures of *S. aureus*, HQNO can induce multidrug tolerance by inhibiting respiration and depleting intracellular ATP[122]. The interference of PYO with respiration in *S. aureus* similarly selects for non-respiring small colony variants[123,126], which are often resistant to antibiotic treatment[127]. In such cases where different secondary metabolites produced by one species seem to have conflicting and condition-dependent effects on a neighboring species, *in vivo* studies and co-culture experiments are particularly necessary to determine the overall impact on clinical antibiotic efficacy.

The potential for *P. aeruginosa*-produced secondary metabolites to have complex effects on antibiotic resilience has also been observed in another opportunistic pathogen, *Stenotrophomonas maltophilia*. Compared to members of the *Burkholderia cepacia* complex, which strongly benefit from exposure to PYO during treatment with ciprofloxacin, *S. maltophilia* is more sensitive to PYO toxicity, exhibiting growth inhibition at concentrations as low as 50 μM[36]. At a low but still lethal dose of ciprofloxacin, PYO at concentrations up to 50 μM statistically significantly increased survival of *S. maltophilia*, which suggests that defenses against fluoroquinolones are indeed induced by PYO in this species[36]. Yet at a ten-fold higher dose of ciprofloxacin, even 10 μM PYO was detrimental[36]. Notably, although *in situ* levels of PYO can vary greatly across patients infected with *P. aeruginosa*, PYO has been detected in infected sputum and wound exudates at concentrations up to 130 μM or 0.31 mg/g, respectively[128,129]. Thus, the example of PYO and *S. maltophilia* suggests that to predict how a secondary metabolite will affect community-wide levels of resilience to clinical antibiotics during a polymicrobial infection, it is imperative to characterize the directionality and magnitude of interspecies effects over a range of clinically relevant concentrations of both the secondary metabolite and the clinical antibiotic.

Implications for resistance evolution

In recent years, it has increasingly become appreciated that antibiotic tolerance cannot only directly contribute to infection treatment failure, but also promote the establishment of heritable resistance mutations[130–134]. Exploration of the connection between antibiotic tolerance and the evolution of resistance has largely focused on tolerance resulting from spontaneous mutations that are selected by treatment with clinical antibiotics[130,131,133,134]. However, effects on the establishment of heritable resistance mutations have also been

demonstrated for at least one secondary metabolite produced by an opportunistic pathogen, namely, PYO. Experiments based on classic fluctuation tests revealed that PYO increases the rate of mutation to antibiotic resistance not only in the producing species, *P. aeruginosa*, but also in a clinical isolate of a co-occurring opportunist, *Burkholderia multivorans*[36]. Notably, the impact of PYO on the acquisition of heritable resistance varied across different classes of antibiotics. In particular, strong effects were observed for drugs against which PYO-induced defenses confer increased tolerance, which suggests that this phenomenon is indeed driven by tolerance as opposed to a mutagenic effect of PYO. Remarkably, in *B. multivorans*, treatment with PYO increased mutation rates for ciprofloxacin resistance to a level rivaling clinical hypermutator strains[36,135]. In addition, in both *P. aeruginosa* and *B. multivorans*, pre-treatment with PYO prior to antibiotic treatment was sufficient to increase the rate at which resistant mutants became established, even without continued exposure to high levels of PYO[36]. These findings collectively reveal the potential for tolerance-inducing secondary metabolites to have a substantial impact on the evolution of antibiotic resistance. However, future experiments will be necessary to investigate the clinical relevance of these observations, and whether the link between tolerance and resistance triggered by PYO exposure can be generalized to other secondary metabolites remains to be determined.

Concluding remarks and future directions

Although direct connections between bacterial secondary metabolite production or exposure and resilience to clinical antibiotics have only been pursued in a small number of studies, many more secondary metabolites are known to interface with cellular functions that are relevant to antibiotic tolerance and resistance, especially drug efflux and oxidative stress responses. We hope the examples discussed in this Review stimulate further investigation into the conditions under which these and related secondary metabolites alter the efficacy of clinical antibiotics, particularly during treatment of infections. Currently, PYO represents the secondary metabolite for which the most detailed evidence on this topic is available, likely due to the extensive research attention that its producer, *P. aeruginosa*, has received. However, we expect that deliberately searching for such molecules across a broad range of opportunistic pathogens (Box 1) will reveal additional as-yet-uncharacterized secondary metabolites that have the potential to affect antibiotic treatment outcomes. Soil-borne opportunistic pathogens in particular often possess the biosynthetic capacity to produce a great variety of secondary metabolites[60,136–138], perhaps because they have to cope with the extraordinary complexity and heterogeneity that typifies the soil environment[139]. In most cases, the biological functions of these secondary metabolites, as well as whether they are produced during infections, remain unknown. However, biosynthetic gene clusters for secondary metabolites are often located near or co-transcribed with genes encoding efflux pumps[140–142], and numerous microbial secondary metabolites are redox-active and therefore have the potential to generate or detoxify ROS[12,143], which suggests that interactions with clinical antibiotic efficacy are likely to be far more common than is currently appreciated.

Importantly, understanding the molecular mechanisms involved in the cellular responses triggered by a secondary metabolite, as well as the chemical properties of the secondary metabolite itself, can provide practical insights regarding which clinical antibiotics are likely to be affected. With this knowledge in hand, combined with an understanding of other

environmental and physiological factors that affect antibiotic susceptibility[144,145], we posit that it will be possible to optimize the use of existing antibiotics so as to better minimize the risk of treatment failure and prevent the evolution of resistance *in vivo*. For example, if a pathogen produces a secondary metabolite that upregulates efflux pumps specific to fluoroquinolones and other aromatic molecules, the chances of successful treatment would likely be higher with another class of antibiotics that is not susceptible to this defense, such as aminoglycosides. The biosynthetic pathways for these secondary metabolites, or even the molecules themselves, could also be targets for the development of new adjuvants for antimicrobial drugs. Such efforts are already underway for secondary metabolites such as PYO and staphyloxanthin[146–149], which are also known to act as virulence factors[150]. For example, enzymatic removal of PYO increased antibiotic killing of *P. aeruginosa* biofilms *in vitro*[149]. In addition, inhibiting a bacterial enzyme that generates H_2S increased the potency of bactericidal antibiotics both *in vitro* and in mouse infection models[151]. These results encourage further exploration of whether targeting the production or presence of specific bacterial metabolites could optimize the clinical efficacy of treatments for infections. Finally, retooling clinical antimicrobial susceptibility testing protocols to account for the impact of secondary metabolites on antibiotic efficacy (Box 2) could potentially improve the predictive value of these assays, especially in the case of secondary metabolite-producing opportunistic pathogens, such as members of the *Burkholderia cepacia* complex, that often exhibit discrepancies between *in vitro* minimum inhibitory concentration measurements and clinical treatment outcomes[152].

In conclusion, our ability to address the vexing challenges posed by antibiotic tolerance and resistance in the future has much to gain by reflecting on the past. The evolutionary and ecological history of natural antibiotics intersects directly with the history of clinical antibiotic discovery. While the soil has continued to provide a rich reservoir for natural product mining efforts, what has gotten lost is the fact that alongside the evolution of pathways that synthesize these molecules, other pathways have co-evolved that respond to them. Remembering this shared historical context is important for predicting how secondary metabolites might affect the response of polymicrobial communities to conventional antibiotics, and compels creative thinking about novel ways to manage such responses.

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Glossary

Tolerance
The ability to survive transient antibiotic exposure

Resistance
The ability to grow in the presence of antibiotics at a given concentration

Antibiotic resilience

The ability of a bacterial population to be refractory to antibiotic treatment via tolerance and/or resistance

Efflux pumps
Membrane-associated transport proteins that are responsible for the extrusion of various compounds out of the cell

Persisters
A subpopulation of bacteria that is killed by a given antibiotic at a much slower rate than the rest of the population, in a manner that is non-heritable

Antioxidant activity
The ability to neutralize highly reactive free radicals

Pro-oxidant activity
The ability to induce oxidative stress

Polymicrobial infection
An infection that is caused by more than one species of microorganism

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Box 1:

Guidelines for establishing causal links between secondary metabolite production and increased antibiotic tolerance or resistance.

We propose a few steps for the investigation of secondary metabolite-mediated changes in antibiotic susceptibility.

Identifying the candidate secondary metabolite.

This can be achieved in multiple ways, including based on molecular structure or its physiological effects on the cells. Genomic analysis could also reveal potentially relevant secondary metabolites produced by pathogens[171]. Researchers should investigate whether the putative secondary metabolite of interest shows any toxicity to the producer, as well as the molecular responses induced upon exposure to it. For example, the fact that pyocyanin (PYO) is toxic to producing cells[35], shares structural similarities to fluoroquinolones (Fig. 1B), and induces efflux systems[9,35], led to predictions about how this metabolite could decrease susceptibility to these drugs[36]. Transcriptomics approaches can be used to reveal responses caused by the specific secondary metabolite[9,27,29,35,36]. Importantly, it will often be necessary to construct a mutant strain lacking the biosynthetic genes for production of the secondary metabolite as a negative control.

Detecting the secondary metabolite.

It is essential to use an accurate and quantitative detection method because the secondary metabolite concentration might affect antibiotic susceptibility. Possible detection methods may include ultraviolet or visible light spectroscopy or mass spectrometry, both of which can be coupled to high-performance liquid chromatography in the case of analyzing complex samples (for example, extracts of microbial cultures or clinical samples). Investigators should attempt to detect the secondary metabolite in the relevant clinical context (for example, in infected sputum, wound exudates or stool samples)[75,76,128,129]. Importantly, concentrations of the secondary metabolite *in vivo* might vary greatly among patients, depending on a variety of infection parameters (for example, infection stage, the strain causing the infection, the number of bacterial cells present). For this reason, the guidelines provided will, at least initially, likely be most useful for cases of chronic infections, where longitudinal monitoring of patients coupled with repeated measurements of the *in vivo* concentrations of secondary metabolites can be used to constrain *in vitro* experiments (see below; and Box 2).

Considering the effects on nearby species.

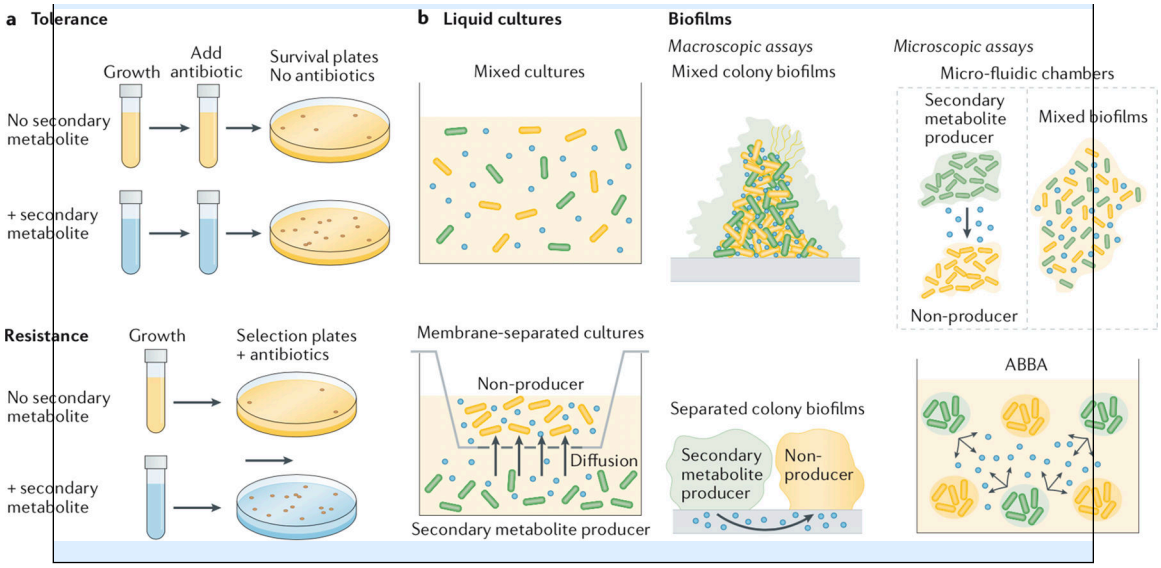
It is also critical to account for the effect of secondary metabolites on the entire microbial community in the case of polymicrobial infections. Thus, investigators should consider which species are most commonly found together with the secondary metabolite producer in the type of infection being studied. Then, investigators should perform experiments in which the producer and non-producers are co-cultured, or the non-producers are separately exposed to controlled concentrations of the secondary metabolite. Understanding how the non-producers are affected, including the molecular

mechanisms involved, is essential for predicting how the secondary metabolite might change the overall antibiotic susceptibility profile of a microbial community.

Testing the secondary metabolite-mediated effects on antibiotic susceptibility.

Tolerance and resistance are two different modes of antibiotic resilience that should be tested separately[14–16] (see the figure, part a); the former can be measured by determining the percentage of surviving cells following a temporary exposure to the antibiotic, whereas the latter calls for assessing the ability to grow in the presence of the antibiotic. There are also various assays available for testing secondary metabolite-mediated effects on drug susceptibility in multispecies communities (see the figure, part b). In liquid co-cultures, the species can be grown with or without separation by a permeable membrane that restricts interactions to those mediated by diffusible small molecules. For biofilms, experiments can be performed in macroscopic assays (for example, colony biofilms, with species mixed or separated), or microscopic assays (for example, microfluidics[172,173] or alternative assays like the agar block biofilm assay (ABBA)[148,174], to which concomitant measurements of microenvironment variables such as pH or O_2 levels have been applied (see the figure, part b)). Importantly, the decision to perform experiments on liquid cultures versus biofilms can influence the results, as some secondary metabolite-mediated tolerance mechanisms are specific to biofilms. For example, the increased release of extracellular DNA mediated by secondary metabolites can stimulate biofilm formation, which results in increased tolerance levels[153]. In addition, redox-active secondary metabolites, such as phenazines, greatly affect metabolism within biofilms[44,175], which in turn modulates antimicrobial drug efficacy[44,74]. By contrast, planktonic cultures provide better control for testing specific secondary metabolite-mediated responses and are amenable to more sophisticated methods when evaluating resistance, such as fluctuation tests, in which a large number of parallel cultures are plated on a selective medium and the number of spontaneous resistant mutants from each culture serves as the input into a mathematical formula for inferring mutation rates[176,177]. The investigators should therefore decide what is more appropriate for their particular questions.

[Figure] The blue color in tubes or plates (in part a) or the blue dots (in part b) represent the secondary metabolite.



Box 2:

Accounting for secondary metabolite production during AST.

As the traditional basis for determining appropriate courses of treatment for infections, antimicrobial susceptibility testing (AST) is a cornerstone of clinical microbiology. However, in many clinical contexts, there is little to no correlation between AST results and treatment outcomes[178,179], which may stem in some cases from the fact that standard AST conditions generally preclude detection of interactions between secondary metabolites and antimicrobial drugs. Clinical AST relies on a readout of absence of growth in cultures that are inoculated at low cell densities[180], whereas secondary metabolites are typically not produced until at least late log-phase or early stationary phase[7]. Moreover, AST is routinely performed on single-species cultures even in the case of polymicrobial infections, eliminating the possibility of detecting secondary metabolite-mediated interspecies interactions that could affect antimicrobial efficacy.

We suggest a few speculative but testable modifications to clinical AST protocols that could help account for the effects of microbial secondary metabolites produced during infections. Our intent is to inspire concrete discussion of how a better understanding of interactions between secondary metabolites and clinical antibiotic efficacy could eventually be applied to improve treatment outcomes, while also recognizing the probable complexity of such endeavors. Given that secondary metabolites can substantially affect antimicrobial efficacy, the proposed modifications might improve the empirical correlation between *in vitro* AST results and the success of clinical treatments. However, many factors beyond secondary metabolite production (for example, biofilm formation, nutritional conditions and/or host-derived molecules) are likely to also affect antibiotic susceptibility *in situ* during infections. The approaches proposed below would need to be refined and validated in animal models of infection, and ultimately tested in clinical trials comparing patient outcomes against the use of traditional AST for antimicrobial drug selection. In addition, if studies indicate that secondary metabolite-related modifications improve the predictive value of AST, scalability in terms of cost and throughput will need to be further optimized before widespread implementation becomes possible in clinical microbiology laboratories.

Use filtered supernatants from overnight cultures of the infective microorganism or microorganisms).

By setting up AST assays using a mixture of fresh growth media and filtered supernatant from an overnight culture of the infective agent, the effects of any secondary metabolites produced during late log phase or stationary phase could be accounted for without needing to modify other aspects of standard AST protocols (for example, visible growth as a readout). A key advantage of this approach is that it is agnostic to which secondary metabolite is produced, avoiding the need for prior knowledge of the biosynthetic capabilities of the infective agent, as well as accounting for the combined effects of multiple secondary metabolites. However, this approach would significantly increase the time to result. In addition, mixing spent supernatant with fresh growth media might dilute the concentrations of the secondary metabolites below clinically relevant levels.

The concentrations of different nutrients in the media would also be affected, possibly in unpredictable ways across different strains, which in turn could also impact antibiotic susceptibility; indeed, the choice of growth medium in general can significantly influence MIC measurements[181].

Add purified secondary metabolites exogenously to cultures.

If it is known that the infective species produces specific secondary metabolites that have been validated as clinically relevant (for example, pyocyanin (PYO) in *Pseudomonas aeruginosa* or indole in *Escherichia coli*), purified forms of the secondary metabolites could be added to traditional AST assays. An advantage of this approach is the ability to control the level of exposure to the secondary metabolites, which would make it possible to ensure that the concentrations in the AST assay are similar to those detected in clinical samples. However, this approach requires that the secondary metabolites be commercially available or otherwise economically viable to synthesize or purify from cultures. In addition, prior knowledge of the biosynthetic capabilities of the infective agent would be necessary. An important caveat in this regard is that microbial secondary metabolite production can vary greatly from strain to strain within the same species. Thus, basing the working concentration of a secondary metabolite on an average across strains or patient samples may lead to over- or underestimation of the effects of the secondary metabolite in individual cases.

Adjust antibiotic ‘breakpoint’ guidelines according to in situ levels of secondary metabolites.

For secondary metabolites that are commonly found in infections and can be procured in purified form, *in vitro* testing could lead to the development of mathematical models that quantitatively relate the concentration of the secondary metabolite to the change in the producing species’ resistance to different antimicrobial drugs. Once validated across a range of different strains, such models could potentially enable secondary metabolite-based adjustments to the standard ‘breakpoint’ antibiotic concentrations used by clinicians to classify microorganisms as susceptible or resistant. Assays for the quantification of the secondary metabolite in patient samples could then be combined with traditional AST testing. Such an approach would enable taking into account strain variability in secondary metabolite production and avoid necessitating modifications to existing AST protocols. However, this approach might also lead to underestimation of secondary metabolite effects in some cases, as bulk measurements of a secondary metabolite in a patient sample may obscure heterogeneity at the micron scale.

Grow multiple species together if they are co-isolated from a polymicrobial infection.

Secreted secondary metabolites produced by one species can affect antimicrobial efficacy in neighboring species. Thus, in the case of polymicrobial infections, inoculating multiple species together in AST assays may improve prediction of the overall community response to antimicrobial drugs. To account for the production of secondary metabolites, this approach would still need to be combined with other modifications, such as those proposed above (points 1–3). In addition, the optimal ratios at which to inoculate

different species would need to be investigated and standardized. Alternatively, it may be possible to perform AST using mixed cultures derived directly from patient samples, either bypassing or in parallel to the step in which individual isolates are obtained and identified.

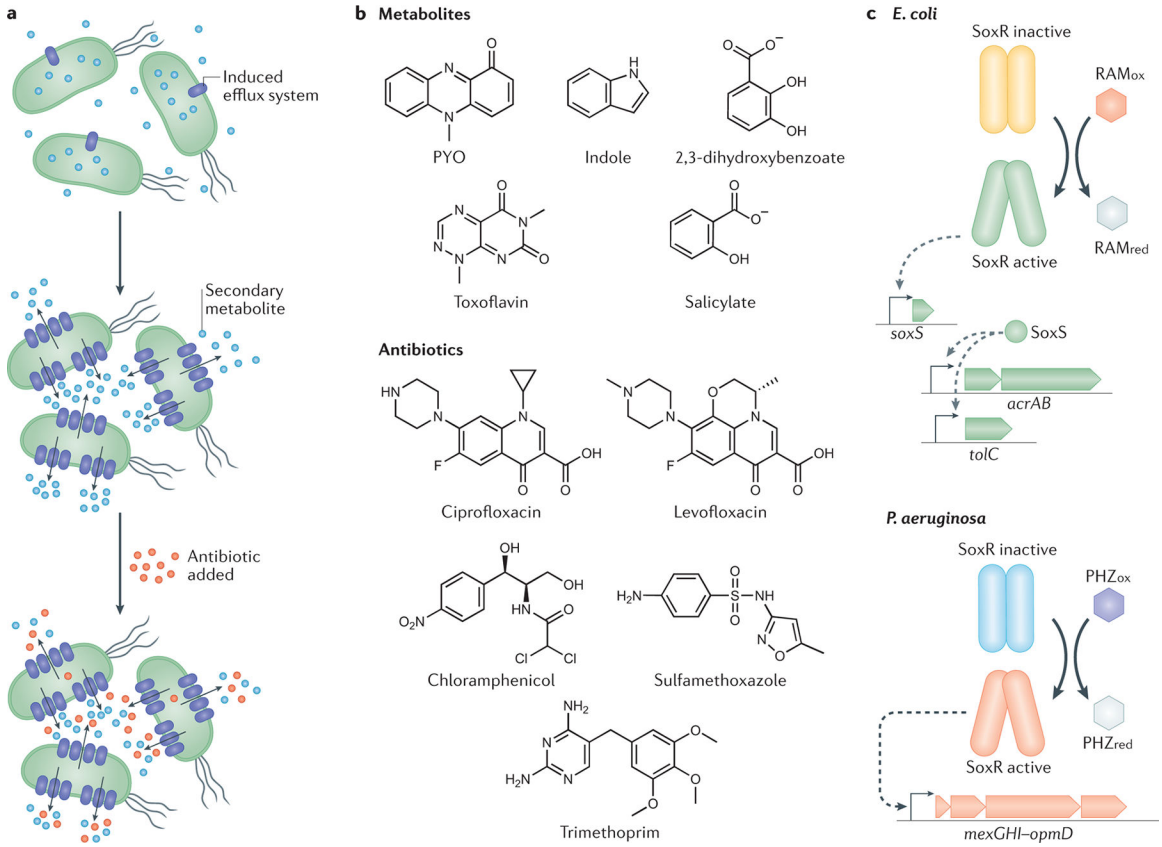


Fig 1. Secondary metabolite-mediated regulation of multidrug resistance efflux pumps.

a. Secondary metabolites induce the expression of efflux systems, which export the metabolite. The increased expression of efflux pumps can provide collateral resilience to antibiotics used in the clinic by expelling the drugs out of the microbial cells. **b.** Structures of known efflux pump-regulating secondary metabolites and selected clinical antibiotics, showing the shared prevalence of aromatic and/or heterocyclic ring motifs. **c.** SoxR-regulated efflux systems in *Escherichia coli* and *Pseudomonas aeruginosa*. Each SoxR monomer contains a Fe–S cluster that can be directly oxidized by redox-active molecules, which leads to its activation and transcriptional induction of efflux systems[162,163]. In *E. coli* (top), several molecules can induce transcription of the efflux system AcrAB–TolC through the activation of SoxR[162,164,165]. Note that *acrAB* and *tolC* are transcribed separately, and this is a simplified version of a very complex regulatory system[22]. Importantly, TolC can assemble with efflux systems from different classes, such as AcrAB–TolC and several others from the resistance-nodulation-division (RND) efflux systems superfamily, EmrAB–TolC (major facilitator superfamily), and MacAB–TolC (ABC superfamily)[23,166,167]. When studying how additional secondary metabolites might affect *E. coli* susceptibility to antibiotics, it will be important to determine which specific efflux system TolC is part of, the regulation involved in the induction of the system, and its substrate-specificity. In *P. aeruginosa* (bottom), the activation of SoxR is mediated by two endogenous phenazines, 5-Me-PCA (not shown) and PYO, and leads to the induction of the efflux system MexGHI–OpmD[9,34,36,163]. PYO, pyocyanin; PHZ_{ox}, phenazine in the oxidized state;

PHZ_{red}, phenazine in the reduced state; RAM_{ox}, redox-active molecule in oxidized state, RAM_{red}, redox-active molecule in reduced state.

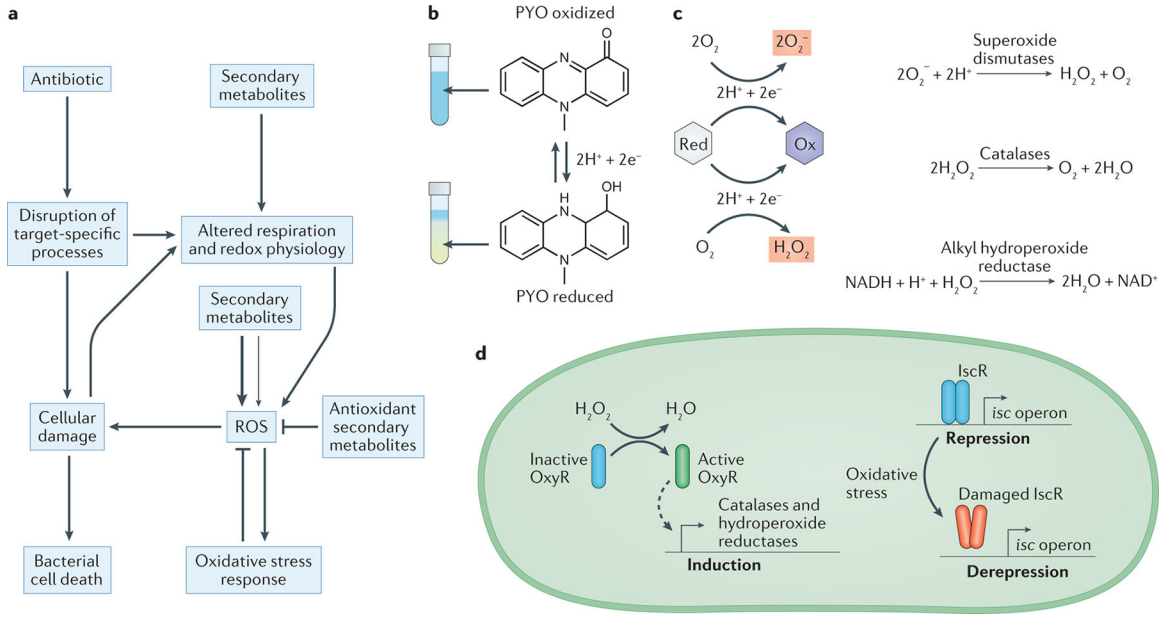


Fig. 2. Secondary metabolite interactions with oxidative stress.

a. Schematic depicting how bactericidal antibiotics can cause cell death both by directly disrupting target-specific processes, and by indirectly promoting the formation of reactive oxygen species (ROS) as a consequence of altered respiration and cellular damage[87]. Secondary metabolites can interface with these pathways at multiple points, including by interfering with respiration and redox homeostasis, directly generating ROS through redox-cycling, and detoxifying ROS via one-electron reactions. Secondary metabolites that promote oxidative stress can either antagonize or potentiate antibiotic toxicity, which is likely to depend on whether the resulting increases in ROS are moderate (thin arrow) or severe (thick arrow). Moderate increases in ROS may induce protective oxidative stress responses that can counteract antibiotic toxicity, whereas severe increases in ROS may overwhelm the defenses of the cell, which leads to synergistic effects with bactericidal antibiotics. **b.** The redox-active nature of pyocyanin (PYO) is visually apparent as it undergoes a color change from blue (oxidized) to colorless (reduced) upon gaining two electrons and two protons from cellular reductants. This reaction is reversible under physiological conditions. **c.** Many redox-active secondary metabolites can donate electrons to molecular oxygen in the process of cycling from a reduced (Red) to an oxidized (Ox) state, which leads to the formation of superoxide or hydrogen peroxide. Cells can detoxify these forms of ROS through enzymatic reactions catalyzed by superoxide dismutase, catalase, and alkyl hydroperoxide reductase[162]. **d.** Bacterial oxidative stress responses are typically regulated through multiple pathways. For example, in *Pseudomonas aeruginosa*, the H_2O_2-sensing transcription factor OxyR controls the expression of catalases and alkyl hydroperoxide reductases (AHPs)[168,169] (left). In addition, the transcription factor iron-sulfur cluster regulator (IscR) upregulates the biosynthesis of iron–sulfur cluster (*isc*) operons in response to oxidative stress caused by ROS such as superoxide or H_2O_2, which can directly damage the iron–sulfur cluster in IscR itself and thereby lead to depression of its regulon[170].

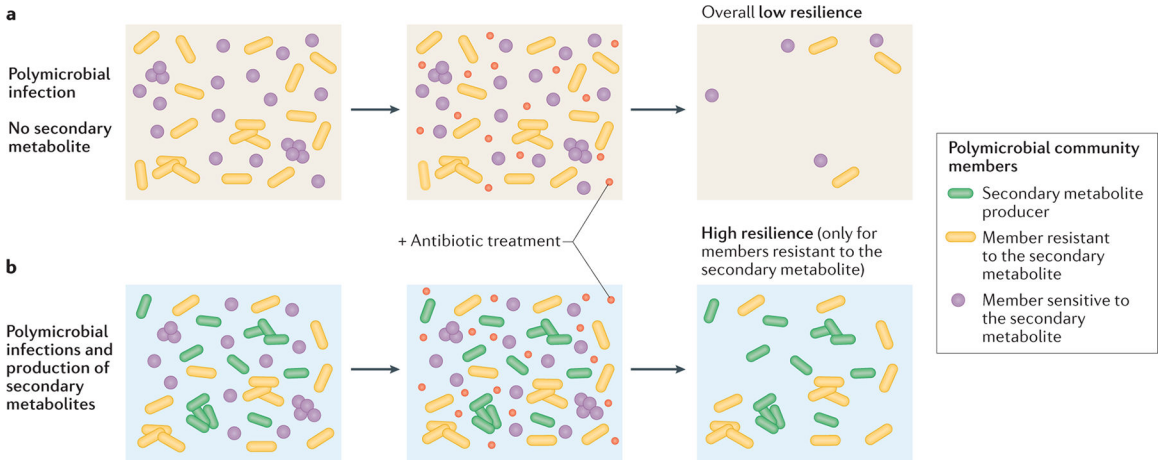


Fig. 3. Secondary metabolites as interspecies modulators of antibiotic resilience.

The presence of secondary metabolite producers in polymicrobial infections can alter the community susceptibility profile to antibiotic treatment. When the producer is not present (part a), overall resilience levels upon antibiotic treatment are low. However, through the secretion of the secondary metabolite, the producer's presence (part b; green cells) can have distinct effects on different community members. For members intrinsically resistant to the secondary metabolite (yellow cells), the molecule's presence can increase resilience to antibiotic treatment. However, if a member is sensitive to the secondary metabolite (purple cells), the added toxicity can overwhelm cellular defenses, potentiating the killing by the clinical drug.

Table 1.

Secondary metabolites produced by opportunistic or enteric pathogens and their impacts on antibiotic efficacy.

Metabolite	Producer	Antibiotic affected	Mechanism	Refs
PYO	*Pseudomonas aeruginosa*	Fluoroquinolones, aminoglycosides*, chloramphenicol, carbenicillin	Efflux induction, oxidative stress response induction	36,44,45
PCA, PCN	*P. aeruginosa*	Ciprofloxacin, tobramycin, carbenicillin	Metabolic changes	44
Paerucumarin	*P. aeruginosa*	Chloramphenicol, ciprofloxacin	Efflux induction	50
Indole	*Escherichia coli*	Fluoroquinolones, gentamicin, ampicillin, carbenicillin	Efflux induction, oxidative stress response induction	27,31,77,111
Salicylate	*Burkholderia* spp.	Chloramphenicol, trimethoprim, ciprofloxacin	Efflux induction	55
HQNO	*P. aeruginosa*	Meropenem	Increased extracellular DNA release and biofilm formation	153
Ergothioneine	*Mycobacterium tuberculosis*	Rifampicin, isoniazid, bedaquiline, clofazimine	Direct ROS detoxification, redox buffering	89
Polyamines (putrescine, spermidine)	*E. coli, Burkholderia cenocepacia, P. aeruginosa*	Levofloxacin, amikacin, cefotaxime, polymyxin B, norfloxacin, rifampicin, tobramycin	Direct ROS detoxification, decreased drug penetration	99,101
PQS	*P. aeruginosa*	Ciprofloxacin, oxofloxacin, imipenem, meropenem, gentamicin, colistin	Increased ROS generation	103,105
H_2S	Diverse microorganisms	Gentamicin, amikacin, nalidixic acid, ciprofloxacin, ampicillin	Oxidative stress response induction, Fe^{2+} sequestration, redox buffering	97,154
Staphyloxanthin	*Staphylococcus aureus*	ND	Direct ROS detoxification	90,91
Carotenoids	*Streptococcus* spp.	ND	Direct ROS detoxification	92,93
2,3-dihydroxybenzoate	*E. coli*	ND	Efflux induction	25
Toxoflavin	*Burkholderia* spp.	ND	Efflux induction**, oxidative stress response induction**	65,68
Phthiocol	*M. tuberculosis*	ND	Oxidative stress response induction**	155,156
D-alanylgriseoluteic acid	*Pantoea agglomerans*	ND	Oxidative stress response induction**	157,158
β−3H-indolydenopyruvate	*Achromobacter* sp.	ND	ND	159
Anthraquinones (for example, emodin, endocrocin)	*Aspergillus* spp.	ND	ND	160,161

HQNO, 2-n-heptyl-4-hydroxyquinoline N oxide; PCA, phenazine 1-carboxylic acid; PCN, phenazine 1-carboxamide; PQS, *Pseudomonas* quinolone signal; PYO, pyocyanin, ROS, reactive oxygen species.

ND, not determined; these are molecules whose effects on antibiotics have not been directly tested.

*For aminoglycosides, PYO has been shown to increase or decrease antibiotic resilience, depending on the studied conditions.

**Hypothesized mechanisms by which the molecule might affect susceptibility.