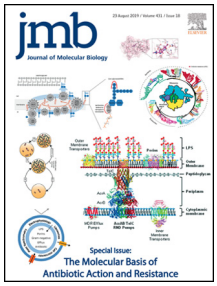

Crossroads of Antibiotic Resistance and Biosynthesis

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Abstract

The biosynthesis of antibiotics and self-protection mechanisms employed by antibiotic producers are an integral part of the growing antibiotic resistance threat. The origins of clinically relevant antibiotic resistance genes found in human pathogens have been traced to ancient microbial producers of antibiotics in natural environments. Widespread and frequent antibiotic use amplifies environmental pools of antibiotic resistance genes and increases the likelihood for the selection of a resistance event in human pathogens. This perspective will provide an overview of the origins of antibiotic resistance to highlight the crossroads of antibiotic biosynthesis and producer self-protection that result in clinically relevant resistance mechanisms. Some case studies of synergistic antibiotic combinations, adjuvants, and hybrid antibiotics will also be presented to show how native antibiotic producers manage the emergence of antibiotic resistance.

Introduction

Antibiotics continue to be the most important resource in the global management of infectious diseases [1,2]. The increased occurrence of antibiotic resistance in human pathogens has raised global concern as antibiotics steadily lose efficacy in clinical and community settings. Despite the rise in antibiotic resistance and shifting drug discovery programs in the private sector, the market for antibiotics remains strong [3,4]. In 2018, the FDA approved four new antibiotics from traditional antibiotic classes including the aminoglycoside plazomicin and tetracyclines eravacycline, omadacycline, and sarecycline [5]. These new antibiotics gained approval, in part, by overcoming established clinical resistance mechanisms to meet a clinical need [6,7]. One can speculate, based on past clinical practice, that new resistance mechanisms will emerge following large-scale deployment of these new drugs (Fig. 1).

Recently, Cox and coworkers [8] performed a detailed investigation of the vulnerability of plazomicin to clinically relevant aminoglycoside inactivating enzymes and ribosomal methyltransferases in a panel of isogenic *Escherichia coli* strains. Plazomicin is a semisynthetic aminoglycoside derived from sisomicin and is designed to chemically block the sites of chemical modification by aminoglycoside inactivating enzymes, including *N*-acetyltransferases (AACs), *O*-nucleotidylyltransferases, and *O*-phosphotransferases (APHs) [9,10]. Indeed, plazomicin was found to maintain activity against *E. coli* expressing the majority of canonical AACs, *O*-nucleotidylyltransferases, and APHs that confer broad-spectrum resistance to several subclasses of aminoglycoside antibiotics [8]. However, plazomicin was covalently inactivated by AAC(2′)-Ia and APH (2″)-IVa with a reduction in growth inhibitory activity against *E. coli* strains expressing these enzymes (Fig. 1). Due to the flexible substrate plasticity and high selective pressure for mutation under plazomicin challenge, it is likely that these enzymes could emerge as real threats after clinical deployment of plazomicin [9,11]. Furthermore, it was discovered that some plasmid-encoded 16S rRNA ribosomal methyltransferases completely abolish plazomicin antibacterial activity, although these resistance elements are still relatively rare [8]. Clearly, the struggle with antibiotic resistance begins far before human use.

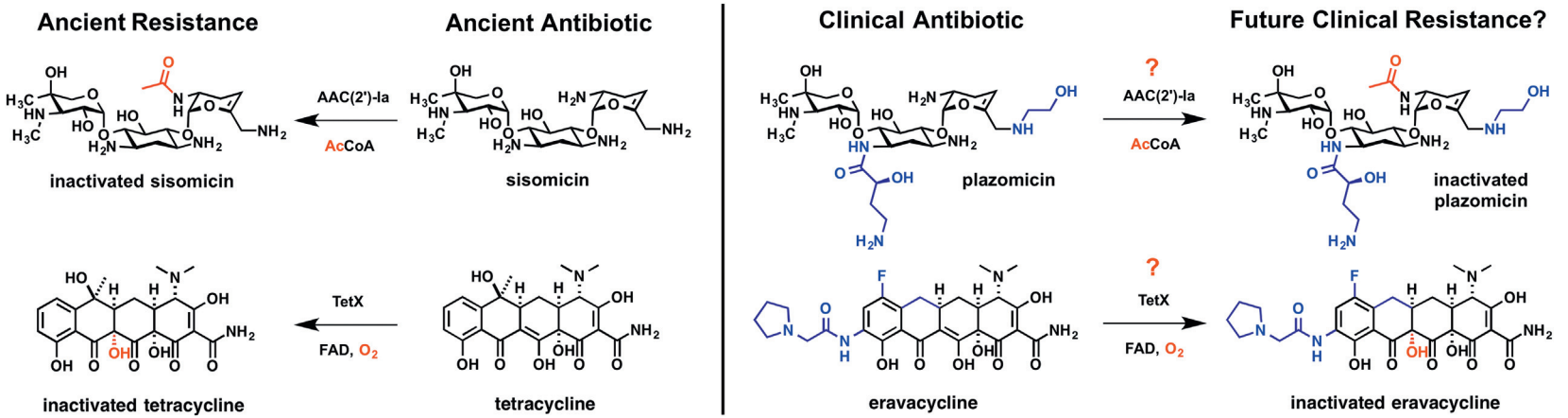


Fig. 1. The ancient antibiotics sisomicin and tetracycline are enzymatically inactivated by aminoglycoside acetyltransferase AAC(2′)-Ia and tetracycline monooxygenase TetX, respectively. The plazomicin and eravacycline scaffolds are derived from the parent sisomicin and tetracycline scaffolds, respectively, and were FDA approved for human use in 2018. Plazomicin and eravacycline have been structurally optimized to overcome some of the established clinical resistance mechanisms for aminoglycoside and tetracycline antibiotics, respectively, and could potentially select for new clinical resistance mechanisms of ancient origin, such as enzymatic inactivation.

Similar to plazomicin, the recently FDA-approved third-generation tetracycline antibiotic eravacycline was strategically designed to overcome traditional resistance mechanisms to tetracycline antibiotics including efflux pumps and ribosome protection proteins [6,7]. Eravacycline also holds the unique place as the only fully synthetic tetracycline to be approved for human use, although it still has the conserved tetracyclic A,B,C,D-ring core of all FDA approved natural and semisynthetic tetracyclines [12]. Eravacycline was evaluated against a panel of isogenic *E. coli* strains expressing efflux pumps (TetK, TetA, TetB), ribosomal protection proteins (TetM), and tetracycline inactivating enzymes (TetX) [7]. Eravacycline maintained nearly full antibacterial activity against *E. coli* expressing TetK, TetA, TetB, and TetM, which represent commonly encountered efflux pumps and ribosomal protection proteins in clinical isolates. It is well known that bulky substituents on the D-ring, a motif common to all third-generation tetracyclines, improves ribosome affinity, blocks the binding of ribosomal protection proteins at the ribosomal A-site, and slows the rate of efflux; so, it is no surprise that eravacycline does the same [13]. However, eravacycline was found to be vulnerable to resistance by TetX, a tetracycline-inactivating enzyme from the flavin monooxygenase (FMO) superfamily [7,14]. *E. coli* expressing TetX showed a 64-fold increase in minimal inhibitory concentration relative to wild-type *E. coli* challenged with eravacycline. TetX has been shown to inactivate first-, second, and third-generation tetracyclines including tigecycline, the first third-generation tetracycline introduced to the clinic [15–17]. Recently, genes encoding TetX along with homologs TetX3 and TetX4 were identified on transferable and inducible plasmids from Enterobacteriaceae and *Acinetobacter* in humans and animals that conferred high levels of resistance to all tetracycline antibiotics including eravacycline and omadacycline [18]. Antibiotic inactivation by TetX, and presumably related homologs, occurs via formation of a C4a-hydroperoxy flavin with subsequent hydroxyl group transfer to C11a of the tetracycline substrate leading to irreversible fragmentation of the tetracycline scaffold [19].

Eravacycline, omadacycline, sarecycline, and future generations of tetracycline antibiotics might fall victim to resistance by enzymatic inactivation (Fig. 1). This lesson has been learned time and again for each new generation of beta-lactam antibiotics introduced to combat widespread inactivation by beta-lactamase resistance enzymes since the first report of a beta-lactamase by Abrahama and Chain in 1940 [20,21]. TetX and related tetracycline inactivating enzymes known as the tetracycline destructases are widely dispersed in natural environments and hospital settings, making this emerging resistance a potential clinical threat [22,23]. The risk for amplifying TetX-like tetracycline inactivating enzymes in human pathogens is a real possibility following widespread use of eravacycline. Widespread distribution of TetX among human pathogens could introduce pan-tetracycline resistant phenotypes that compromise the future use of the entire antibiotic class. Thus, it is imperative to develop resistance management plans proactively, including next-generation tetracyclines that overcome inactivation by TetX and adjuvants that inhibit TetX-mediated degradation of tetracycline antibiotics [24,25]. The freedom to fully control the eravacycline scaffold through total synthesis and advances in synthetic biology will greatly enhance the production of next-generation analogs and inhibitors to keep pace with emerging resistance [12,26]. Continuous evolution of beta-lactam antibiotic scaffolds through chemical and biochemical means has proved paramount for maintaining clinical efficacy of this critical drug class [27].

Lessons learned from >80 years of clinical antibiotic use and development have provided a roadmap for the mechanisms, emergence, and dissemination of antibiotic resistance in hospital and community settings [28]. Scientific advancements from the genomic era have been especially important for making the connections between antibiotic resistance in environmental microbes and human pathogens. The vast majority of clinical antibiotics are derived from microbial natural products [29]. Thus, the vast majority of clinical antibiotic resistance originates from natural environments where antibiotic biosynthesis is prevalent [30]. In fact, the aminoglycoside modifying and tetracycline inactivating enzymes discussed previously are likely to have origins in the biosynthesis of aminoglycoside and tetracycline antibiotics, respectively [31–33]. The crossroads of antibiotic biosynthesis and resistance are fertile ground for microbial evolution. Antibiotic resistance has reached equilibrium in well-established natural ecosystems where antibiotic-resistant and antibiotic-sensitive organisms enjoy mutually beneficial lifestyles in the presence of diverse antibiotic cocktails [30,34,35]. The evolutionary pressure from humans in the balance of antibiotic resistance is best appreciated in arenas where large-scale deployment of antibiotics is used for health and economic benefits, namely, in hospital and agricultural settings [36]. Unlike natural environments where microbes produce low concentrations of multiple antibiotics, humans tend to deploy large concentrations of single antibiotics for desired applications [37]. This is best practice for the selection of highly resistant phenotypes, including multi-drug resistance (MDR) to mechanistically related clinical antibiotics such as observed for the well-known macrolide-lincosamide-streptogramin phenotypes for gram-positive human pathogens resulting from expression of ribosome-modifying methyltransferases that perturb common antibiotic

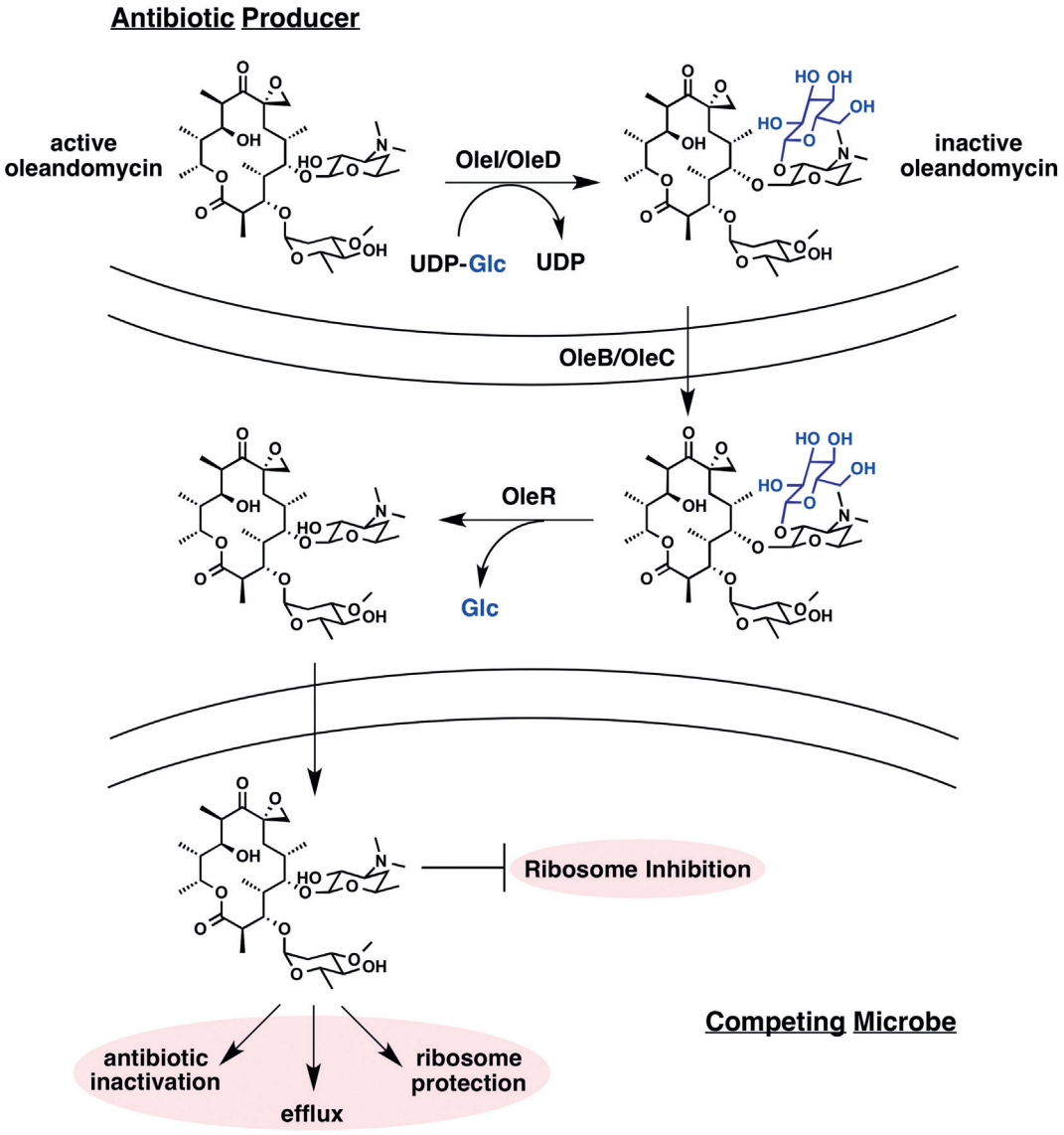


Fig. 2. Macrolide resistance in producers and competing microbes.

binding sites around the peptidyl transferase center [38].

The exact trajectory of gene transfer for any given antibiotic resistance event is difficult to delineate [28,30]. There are known and speculated hotspots for enriched pools of antibiotic resistance genes (ARGs) including wastewater treatment plants, antibiotic manufacturing sites, agricultural sites employing antibiotic feedstock, and hospitals. The vectors for exchange of ARGs are thought to be genetically competent opportunistic human pathogens that readily grow in natural environments where antibiotics are prevalent. The individual ARGs representing the full spectrum of antibiotic resistance mechanisms (efflux, exclusion, target modification, sequestration, covalent inactivation) originate from selective pressure where self-protection genes that provide producers innate resistance are transferred between environmental microbes via mobile genetic elements [39]. Thus, studying the biosynthetic origins of antibiotics provides insight into potential resistance mechanisms that currently exist or might emerge upon clinical use of the antibiotic or a derivative thereof. Proactive investigation of dormant and emerging resistance mechanisms is further merited because environmental microbes often provide natural strategies for overcoming resistance associated with a given antibiotic in the form of adjuvants, synergistic antibiotic combinations, and pro-drug formulations. Comprehensive reviews on the mechanisms, origins, and dissemination of antibiotic resistance are readily available [30,40]. More recently, Almabruk and coworkers [41] provided a survey with case studies on the topic of self-

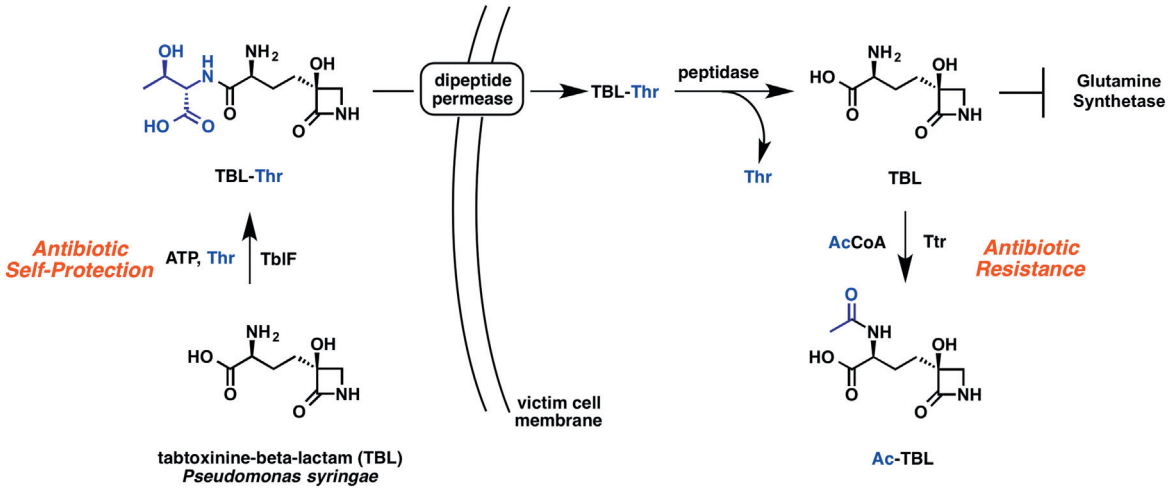


Fig. 3. Reversible modification of TBL by amino acid ligase TblF to form the pro-drug TBL–Thr dipeptide in *P. syringae*. Irreversible modification of TBL by GNAT acetyltransferase Ttr confers resistance in competing microbes.

protection by antibiotic producing microbes. Here, we aim to provide a more focused perspective on the origins, mechanisms, and evolutionary trajectory of antibiotic resistance in the form of enzymatic inactivation to highlight crossroads with antibiotic biosynthesis that inform new methods for predicting and managing emerging clinical resistance mechanisms in human pathogens.

Antibiotic Resistance and Producer Self-protection

Self-protection is a pre-requisite for any antibiotic-producing microbe [42–44]. It is common for antibiotic biosynthetic gene clusters (BGCs) to contain genes encoding the enzymes required for both biosynthetic assembly of the antibiotic scaffold and self-protection mechanisms [41]. This co-clustering leads to co-expression of genes and ensures self-protection during production of the toxic antibiotic. Often BGCs also contain genes associated with quorum sensing regulation to ensure timely antibiotic production in mixed microbial environments where community metabolism is at play [45]. Self-protection by antibiotic producers is often referred to as *innate* resistance, while the appearance of self-protection genes in non-antibiotic producers is known as *acquired* resistance.

Typically, what is classified as authentic clinical resistance in human pathogens falls under the category of *acquired* resistance; however, human pathogens are often innately resistant to certain antibiotics. External gene acquisition is not a strict requirement for *acquired* resistance mechanisms. In addition, resistance events caused by single nucleotide polymorphisms resulting in amino acid point mutations in the antibiotic target protein would fall under the category of *spontaneous* resistance. This type of resistance is commonly observed when human pathogens are treated with small-molecule antibiotics including natural products. A serial passage experiment with increasing dose of the antibiotic can often reveal the molecular target of a given antibiotic through genome sequencing to locate the sites of resistance-conferring mutations [46].

Innate resistance in the form of self-protection during antibiotic production does not always require a set of dedicated ARGs. For example, *Eleftheria terrae* is a gram-negative member of a new genus related to *Aquabacteria* that produces a highly potent lipid I–III sequestering antibiotic known as teixobactin [47,48]. This molecule rose to fame in 2015 among claims of no “detectable resistance,” an apparent contradiction for any antibiotic. Teixobactin is produced via non-ribosomal peptide synthetase (NRPS) enzymes in the *E. terrae* cytoplasm followed by export to the extracellular space via an inner-membrane/outer-membrane spanning RND family efflux pump encoded by genes in the teixobactin BGC. Gram-negative bacteria, including the producer *E. terrae*, are *innately* resistant to NRP antibiotics targeting lipids I–III, including teixobactin and vancomycin, that fail to permeate the outer lipid membrane that shields the lipid I–III cell membrane components localized to the inner lipid membrane [49]. In gram-positive bacteria, the lipid I–III molecules are left exposed on the extracelluar surface of the lipid membrane where complex formation with teixobactin can occur to halt peptidoglycan assembly and induce a futile cycle leading to cell lysis.

Both antibiotic producers and non-producers are capable of achieving *innate*, *acquired*, and *spontaneous* types of antibiotic resistance. BGCs in antibiotic producing microbes are a long-term

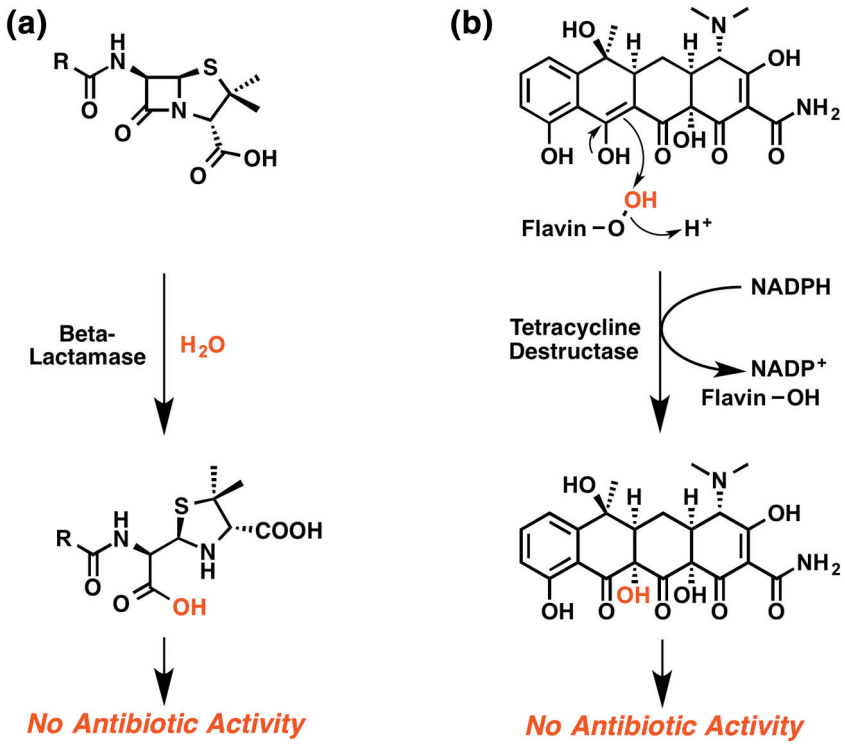


Fig. 4. Resistance evolves with chemistry. (a) Hydrolysis of strained beta-lactam ring in penicillin by a beta-lactamase. (b) Oxidation of electron rich C11a-enol in tetracycline by a tetracycline destructase C4a-flavin peroxy intermediate.

repository for ARGs [34]. Genetic mobilization of these ARGs creates an on-demand supply for mixed microbiomes that can lead to dissemination into human pathogens [28,30]. The complete spectrum of antibiotic resistance mechanisms (efflux, exclusion, target modification, sequestration, enzymatic inactivation) is represented in the self-protection strategies invoked by antibiotic producing microbes [40]. A classic example of this multi-pronged approach is found in *Streptomyces antibioticus*, a natural producer of the macrolide antibiotic oleandomycin (Fig. 2) [50]. Oleandomycin is kept in an inactive glycosylated form in the *S. antibioticus* cytoplasm via two glycosyl transferases, OleI and OleD, encoded in the oleandomycin BGC [51]. OleI is specific for oleandomycin, while OleD shows a broader substrate scope hinting at its role as a general macrolide inactivating resistance enzyme [52]. Glycosylated oleandomycin is excreted to the extracellular space in pro-drug form via the OleB/OleC efflux pumps. An excreted glycosyl hydrolase, OleR, reveals the active protein synthesis inhibitor safely beyond the reach of the cytoplasmic ribosome target of the producer. Competing microbes that ingest oleandomycin are susceptible to ribosome inhibition allowing the producer a competitive growth advantage. These competing microbes still have opportunities for resistance gain-of-function through acquisition of efflux pumps, ribosome methyltransferases, and/or macrolide-inactivating enzymes including glycosyl transferases related to OleI and OleD [38,53].

Glycosylation of oleandomycin represents a model for the co-evolution of substrate specificity and plasticity into antibiotic inactivating enzymes [52]. The reversible glycosylation of oleandomycin by the glycosyl transferase OleI and secreted glycosyl hydrolase OleR represents a useful *self-protection strategy* in *S. antibioticus* that preserves the antibiotic payload enabling dual use as a *pro-drug strategy*. Expression of OleD ensures survival in the presence of exogenous macrolides produced by competing microbes and represents a useful *resistance strategy* that can be passed on to human pathogens resulting in pan resistance to clinical macrolides [53]. The complex interplay between producer immunity and resistance evolution through microbial competition provides the chemical breeding grounds for evolving antibiotic inactivating enzymes.

Origins of Antibiotic Inactivating Enzymes

The most potent form of antibiotic resistance comes in the form of enzymatic inactivation [40,54,55]. Unlike other resistance mechanisms,

covalent modification of antibiotics depletes the total concentration of the antibiotic challenge below inhibitory levels rather than controlling local concentrations (intracellular *versus* extracellular) through exclusion, efflux, and sequestration or modifying the target to tolerate higher antibiotic concentrations. The flux of enzymatic antibiotic construction (biosynthesis) and destruction (self-protection/resistance) influences microbial population dynamics and drives the emergence of antibiotic inactivating enzymes.

Reversibility of antibiotic inactivation

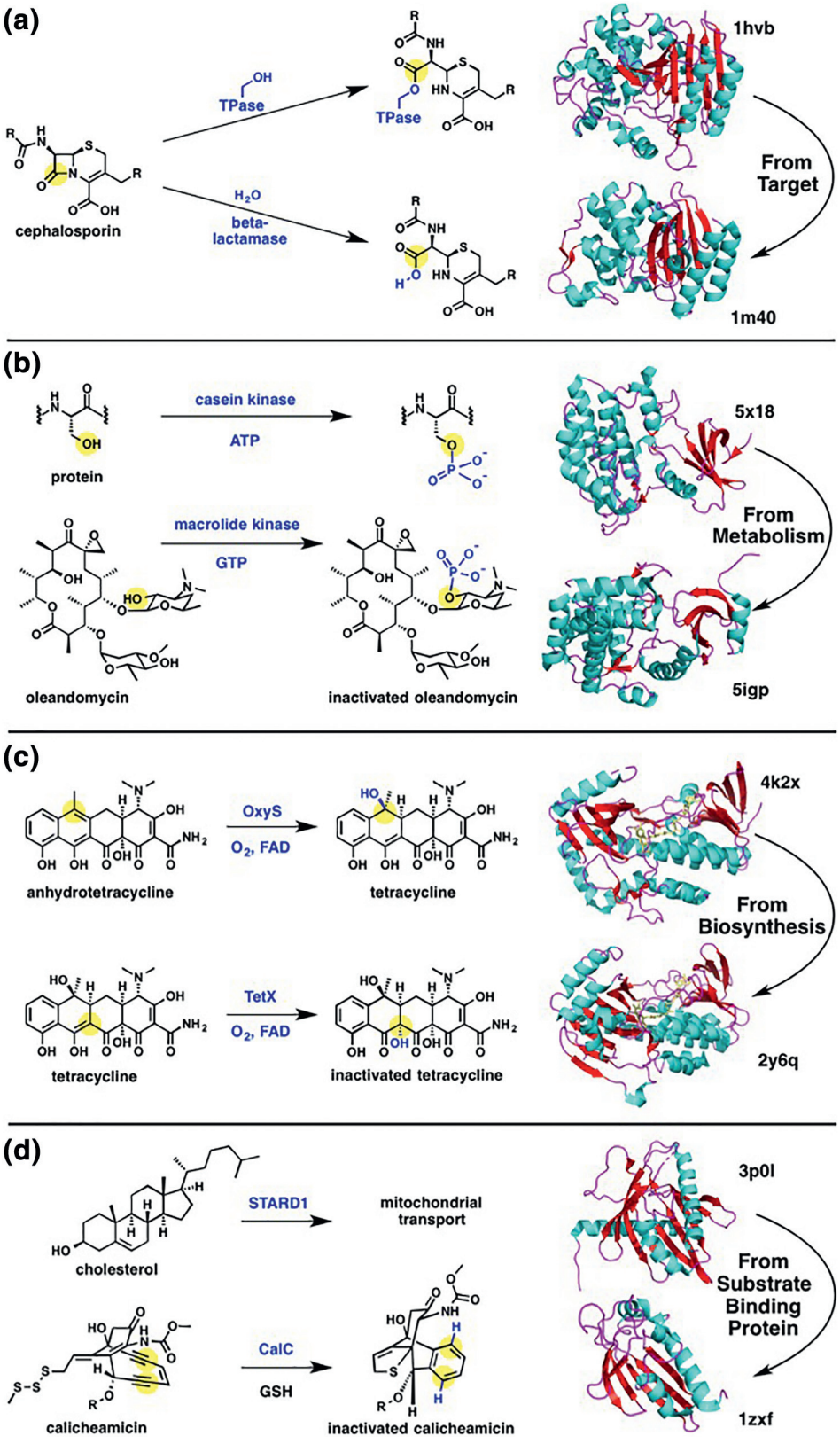
Antibiotic inactivating enzymes provide cellular immunity by modifying a portion of the antibiotic scaffold that is required for target binding. Differences arise in the types of chemical bonds formed when comparing producer self-protection and resistance in clinical pathogens. Antibiotic inactivation by self-protection enzymes tends to be *reversible*, while inactivation by resistance enzymes tends to be *irreversible* on a biologically relevant time scale. For example, consider the glutamine synthetase (GS) inhibitor tabtoxinine-beta-lactam (TBL) produced by plant pathogenic strains of *Pseudomonas syringae* (Fig. 3) [56]. The alpha-carboxyl group of TBL is converted to the corresponding L-Thr dipeptide by the ATP-dependent L-Thr ligase TblF to facilitate efflux by TblR to the periplasm away from cytoplasmic GS [57]. The TBL–Thr dipeptide is effluxed to the extracellular space where competing microbes can import the pro-drug via surface-displayed dipeptide permeases. Cytoplasmic dipeptidases can hydrolyze the TBL–Thr dipeptide revealing the GS inhibitor TBL in the cytoplasm where GS inhibition can be achieved [58]. If intracellular TBL concentrations become too high, *P. syringae* can produce the Gcn5-related *N*-acetyltransferase (GNAT) Ttr that inactivates TBL via acetylation of the alpha-amino group [57]. Both amidation of the TBL alpha-carboxylate by TblF and acetylation of the TBL alpha-amino group by Ttr result in covalent modifications that prevent GS binding. However, the amidation of TBL is *reversible*, while acetylation appears to be *irreversible*. For this reason, only heterologous expression of Ttr, not TblF, in *E. coli* confers resistance to TBL–Thr [58]. Dipeptide formation is a common pro-drug approach for non-proteinogenic amino acid antimetabolites including phosphinothricin, alaphosphin, and many more [59,60]. Phosphinothricin is a dipeptide pro-drug of the phosphinate-based GS inhibitor glufosinate and *Streptomyces* producer self-protection is achieved by expression of the *bar* gene, a GNAT related to Ttr in structure and function [61]. Transgenic plants expressing the *bar* gene are resistant to glufosinate and form the basis of a broad-spectrum herbicide strategy marketed by Bayer under the trade name LibertyLink® [62]. This is a case where *irreversible* antibiotic inactivation can be used to benefit society through application to agricultural chemistry.

Thermodynamic and kinetic considerations

Antibiotic resistance by enzymatic inactivation evolves with chemistry. The chemical reactions catalyzed by antibiotic inactivating enzymes tend to exploit the weakest link in the antibiotic scaffold. Consider beta-lactam antibiotics: all contain the strained 2-azetidonine heterocycle (a.k.a. beta-lactam) as the source of antibacterial activity [63]. Beta-lactams are kinetically stable, but thermodynamically labile and are susceptible to *irreversible hydrolysis* of the beta-lactam ring (Fig. 4a). Beta-lactamase enzymes catalyze the *irreversible hydrolysis* of 2-azetidinones with fast kinetics that can approach diffusion-controlled efficiency for some beta-lactam subclasses [21,64]. Each structural class of antibiotic possesses unique reactivity exploited by inactivating enzymes. For example, tetracycline antibiotics are not readily susceptible to hydrolysis like beta-lactams but the electron rich extended pi-conjugation of the tetracyclic core is naturally susceptible to photooxidation and chemical oxidation [14]. It follows that a class of FMO enzymes have emerged as tetracycline inactivating enzymes [22]. These tetracycline inactivating FMOs catalyze the *irreversible oxidation* of tetracycline antibiotics via formation of a reactive C4a-flavin peroxy intermediate (Fig. 4b) [19].

Evolution from the biological target

The emergence of antibiotic inactivating enzymes can follow distinct evolutionary paths. In the case of beta-lactamases, at least four distinct evolutionary classes have emerged (A–D) [65]. The most common class A beta-lactamases, including TEM-1, are serine hydrolases *evolved directly from the target* D,D-transpeptidase domain of the bacterial penicillin-binding proteins (PBPs) (Fig. 5a) [66]. The primary differences between beta-lactamases and PBPs arise in the kinetics of the active site serine deacylation step, which in comparison is fast for beta-lactamses and slow for PBPs [21,67] The emergence of extended spectrum beta-lactamases followed shortly after widespread distribution of plasmid-encoded TEM and SHV beta-lactamases through gain-of-function mutations of the parent ARG [64]. Extended-spectrum beta-lactamases benefit from an expanded substrate tolerance and improved catalytic efficiencies. Such enhancements in substrate plasticity can be recreated in the laboratory setting through directed evolution experiments, as discussed in a later section [68].



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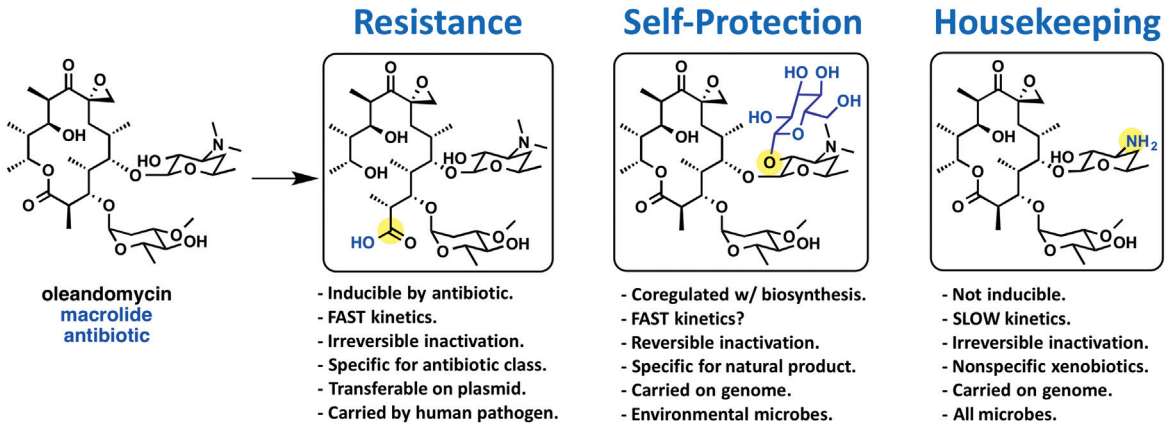

Fig. 6. Comparison of macrolide inactivating enzymes used for resistance (macrolide esterase), self-protection (macrolide glycosyltransferase), and general metabolic housekeeping (demethylase) in microbes. Chemical modifications of the macrolide erythromycin are highlighted in blue with the site of functional group modification highlighted by a yellow circle.

Evolution from general metabolism

Mutation of the target enzyme into an antibiotic destructase seems plausible for cases where suicide inhibition (inhibition of the enzyme at the expense of the inhibitor scaffold) is at play, as is true for the beta-lactam antibiotics. For antibiotics where target inhibition is non-covalent, it is possible for *enzymes from general metabolism* to emerge as antibiotic destructases. Both housekeeping metabolic enzymes and antibiotic inactivating enzymes covalently modify substrates, but there are a few noteworthy differences (Fig. 6) [52,69,70]. Housekeeping enzymes tend to have broad substrate plasticity, exhibit slow kinetics, and are expressed constitutively. In some cases, antibiotic inactivating enzymes are highly selective for a specific structural class of antibiotic substrates with broad substrate plasticity within the antibiotic class, exhibit fast kinetics, and are inducible by the antibiotic challenge. Furthermore, ARGs can be plasmid-encoded reflecting genetic mobility under selective pressure applied by antibiotic exposure while housekeeping genes are carried on the chromosome. It should be noted that housekeeping and ARGs are not necessarily mutually exclusive as many bacteria harbor species-specific chromosomal beta-lactamases [21].

Pawlowski and coworkers [71] recently demonstrated how substrate specificity emerges in a family of GTP-dependent macrolide inactivating kinases (Mph) using ancestral sequence reconstruction combined with directed evolution and functional selections. The Mph kinases phosphorylate the conserved 2′-OH group of the dimethylamino sugar to prevent ribosome binding and confer resistance to Mph producers [72,73]. Similar kinases have emerged as resistance enzymes against aminoglycoside and tuberactinomycin antibiotics, and all seem to have a structural relationship to eukaryotic protein kinases supported by the retention of residual, albeit weak, Ser-protein kinase activity (Fig. 5b) [74,75]. Narrow-spectrum Mphs were found to be common in Bacillales with catalytic phosphorylation rates against clinical macrolides being too low to confer resistance [71]. In the Wright study, four residues were logically connected to expanding substrate accommodation in Mphs, while several less obvious mutations were shown to improve catalytic efficiency to provide gain-of-function resistance when expressed in a heterologous host. This approach for ancestral sequence reconstruction will likely be generally applicable to antibiotic inactivating enzymes that are amenable to gain-of-function via directed evolution including beta-lactamases [68], tetracycline FMOs [15], and aminoglycoside phosphotransferases [76,77].

Evolution from a biosynthetic enzyme

While macrolide phosphorylation does appear to be a sufficiently irreversible modification to confer resistance, a more effective resistance strategy would seem to be the destructive hydrolysis of the macrolactone ring common to the entire antibiotic class. In fact, plasmid encoded macrolide esterases,

Fig. 5. Evolution of antibiotic inactivating enzymes. (a) Evolution of beta-lactamases from target D,D-transpeptidases. (b) Evolution of protein kinase into a macrolide inactivating enzyme. (c) Evolution of a tetracycline inactivating enzyme ("antibiotic destructase") from a biosynthetic FMO ("antibiotic constructase"). (d) Evolution of an enediyne self-sacrificing protein from a substrate binding protein.

Table 1. Functional activity and evolutionary origins of representative antibiotic modifying enzymes

Antibiotic destructases		Susceptible functional groups	Relevant antibiotic classes	Representative antibiotics	Natural role	References
Hydrolases	Beta-lactamases	Beta-lactam ring, beta-lactone ring	Beta-lactams, beta-lactones	Amoxicillin, cephamycin, thienamycin, aztreonam, obafluorin	Self-protection	[65,84]
	Esterases	Esters, macrolactones	Macrolides	Erythromycin, oleandomycin	Unknown	[69]
	D,D-Peptidases	Peptide bonds	Non-ribosomal peptides	Bogorol, bacitracin, zwittermicin, colibactin, xenocoumacin	Self-protection/pro-drug release	[85–88]
	Cyclopropane hydrolase	Cyclopropane ring	Cyclopropane antibiotics	Colibactin	Self-protection	[89]
	Epoxide hydrolase	Epoxides	Epoxide antibiotics	Fosfomycin	Unknown	[90]
	Di-/tripeptidase	Peptide bond	Di- and tripeptides	Phosphinothricin, tabtoxin, bacilysin	Prodrug release	[91]
	Glycosidase	Glycosidic linkage	Macrolides	Oleandomycin	Prodrug release	[50–52]
	Phosphatase	Phosphoester	Aminoglycosides	Streptomycin	Prodrug release	[31,92]
	Amidase	Amide	Amphenicols, beta-lactams	Chloramphenicol, penicillin	Self-protection, antibiotic catabolism	[93–95]
	Thioltransferases	Epoxides, ketones, aldehydes, michael acceptors	Fosfomycin, abyssomicin	Fosfomycin, abyssomicin	Xenobiotic detoxification	[96–98]
Transferases	Phosphotransferases	Hydroxyl groups	Aminoglycosides, macrolides, amphenicols, fosfomycin, ansamycins	Kanamycin, erythromicin, chloramphenicol, fosfomycin, rifampin	Self-protection/pro-drug formation	[11,53,71,72,74, 76,99–103]
	Acetyltransferases	Hydroxyl groups, amino groups	Streptogramins, antimetabolites, amphenicols, aminoglycosides, fluoroquinolones	Virginiamycin, TBL, azetidine-2-carboxylate, chloramphenicol, kanamycin, ciprofloxacin	Self-protection, antibiotic catabolism	[57,61,104–113]
	Glycosyltransferases	Hydroxyl groups	Macrolides, ansamycins	Oleandomycin, erythromicin, rifamycin	Self-protection/pro-drug formation	[52,53,114]
	Methyltransferases	Sulfhydryl, amino, and hydroxyl groups	Dithiolpyrrolones, dithioldiketopiperazines	Holomycin, gliotoxin	Self-protection	[115,116]
	Nucleotidylyltransferases	Hydroxyl groups	Aminoglycosides, lincosamides	Kanamycin, clindamycin	Self-protection	[11,76,117–120]
	ADP-ribosyltransferases	Hydroxyl groups	Ansamycins	Rifamycin	Self-protection	[121,122]
	Amino acid ligase	Amino acids	Amino acid antimetabolites	Phosphinothricin, tabtoxin, alaphosphin	Prodrug formation	[57,59,60]
	Asparagine acyl transferase	Asparagine amino group	Non-ribosomal peptides	Zwittermicin, colibactin, xenocoumacin	Prodrug formation	[86–88]
Lyases	Streptogramin lyase	Esters	Streptogramins	Virginiamycin, quinupristin	Self-protection	[123,124]
Oxido-reductases	Flavin monooxygenases	Electron-rich olefins, electron-deficient ketones	Tetracyclines, ansamycins	Tigecycline, rifamycin	Biosynthesis/self-protection	[14,17,22, 125,126]
	Quinone reductase	Quinones	Mitomycins	Mitomycin C	Self-protection/pro-drug	[127]
	Nitro reductase	Nitro groups	Amphenicols	Chloramphenicol	Xenobiotic detoxification	[128,129]
	Ketoreductase	Ketone	Streptogramins	Virginiamycin M_1	Self-protection	[130]
Self-sacrificing proteins	Substrate-binding proteins	Chemical sequestration	Enediynes, albicidin	Dynemicin, albicidin	Self-protection	[81–83]
	Glycyl cleavage proteins	Enediyne	Enediynes	Calicheamicin	Self-protection	[80]

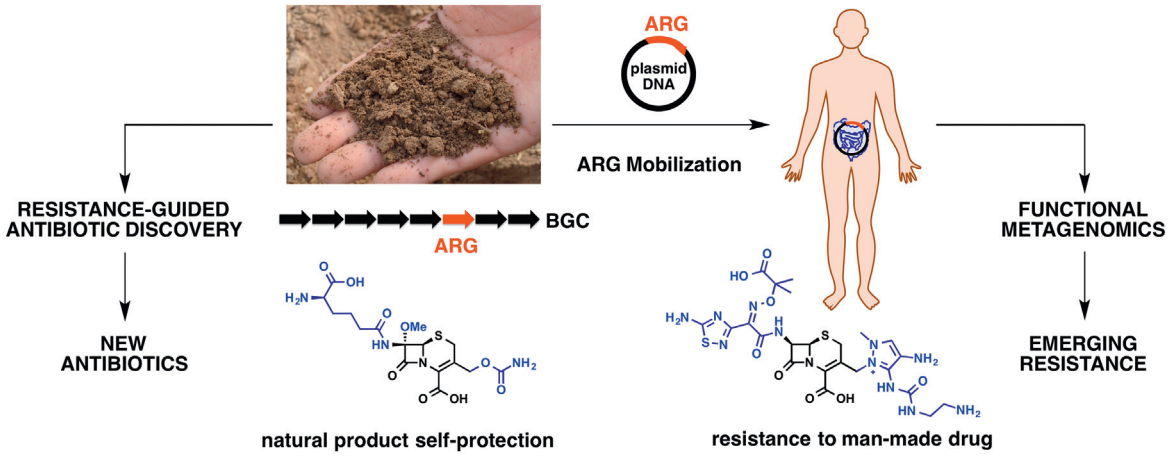

Fig. 7. Dissemination of ARGs from soil to the human gut. Ancient antibiotic resistance to the natural cephalosporin can be a source of resistance to the man-made cephalosporin used in hospitals when ARGs from a BGC are mobilized on plasmids. This natural phenomenon can be leveraged for resistance-guided antibiotic discovery (left) to prospect for new antibiotics and functional metagenomic screens (right) to prospect for emerging resistance. The image of soil was obtained from pixabay.com and is free for use in the public domain.

including erythromycin esterase EreA, have been discovered in clinical pathogens (Fig. 6) [53,69]. To date, no structure of a macrolide esterase has been solved. Sequence comparison and homology modeling suggests that macrolide esterases (EreA, EreA2, EreB, EreC, and EreD) are metalloenzymes predicted to be structurally related to esterases involved in succinoglycan biosynthesis (BcR135 and BcR136). It is intriguing that macrolides are products of type I polyketide synthases where product release from the enzymatic assembly line is facilitated by a C-terminal hydrolase domain that catalyzes macrolactonization as opposed to hydrolysis of the intermediate acyl enzyme oxoester intermediate [78]. It is conceivable that these macrolactonization catalytic domains could also evolve into autonomous macrolide esterases through accommodation of a nucleophilic water in the active site. Such conversion of an "antibiotic constructase" to an "antibiotic destructase" might be favorable since the ancestral biosynthetic enzyme has already evolved the substrate-binding pocket and catalyzes the necessary chemistry for antibiotic inactivation (lactonization *versus* hydrolysis in the case of macrolides). Based on the ancestral sequence reconstruction and directed evolution approach applied toward other classes of antibiotic inactivating enzymes, the acquisition of gain-of-function mutations in *ancestral biosynthetic enzymes* that confer resistance through antibiotic inactivation seems plausible [71].

Another case for emergence of an "antibiotic destructase" from an "antibiotic constructase" might be found in the tetracycline FMO family of tetracycline inactivating enzymes (Fig. 5C) [22]. Oxidation of the anhydrotetracycline C-ring at carbon-6 by an FMO, OxyS, provides the mature tetracycline scaffold during biosynthetic assembly [32,33]. The tetracycline FMOs are more versatile and oxidize tetracyclines at a variety of positions including enol C11a and ketone C12 leading to oxidized degradation products via hydroxylation or Baeyer–Villiger oxygen insertion, respectively, that lack antibacterial activity [19]. Some tetracycline FMOs, including TetX, can also oxidize anhydrotetracycline and show broad substrate plasticity within the tetracycline antibiotic class that rivals clinically prevalent antibiotic inactivating enzymes including macrolide and aminoglycoside kinases [14,24,25]. Similar to macrolide kinases, TetX readily accepts gain-of-function mutations via directed evolutionary pressure that enhance catalytic efficiency toward the oxidative inactivation of third-generation tetracyclines including tigecycline, eravacycline, and omadacycline [15,18]. The evolutionary trajectory from OxyS or related FMOs to tetracycline destructases has not been fully explored.

Evolution from a substrate-binding protein

Yet another evolutionary line of antibiotic inactivation has emerged from *substrate-binding proteins* (SBPs) (Fig. 5d). SBPs are typically considered noncatalytic proteins that reversibly bind small molecules with high affinity [79]. A somewhat odd case has emerged where an SBP encoded in BGCs for enediyne natural products can act either as a sequestering agent to prevent association with target DNA [80] or a single turnover catalyst to quench reactive para-benzyne radicals generated from protein sequestered parent enediyne through C-H abstraction from a conserved glycine residue leading to protein fragmentation [81,82]. BGCs for

anthraquinone-fused 9-membered enediynes such as dynemicin seem to be rich in SBPs such as TnmS1, TnmS2, and TnmS3 that non-covalently sequester the DNA-damaging enediynes from DNA in the cytoplasm to ensure self-protection [80]. Recently, the x-ray structure of the DNA gyrase inhibitor albicidin bound to a BGC-encoded sequestering protein AlbA was solved showing how high-affinity cytoplasmic sequestration can be an effective self-protection strategy [83]. The calicheamicin BGC and related 10-membered enediynes are also DNA-damaging agents with mechanism of action similar to the 9-membered enediynes. Calicheamicin is a prodrug where activation is triggered via disulfide reduction leading to formation of a reactive para-benzyne radical capable of DNA cleavage chemistry. Calicheamicin producers encode self-sacrificing SBPs such as CalC that act as single turnover catalysts to inactivate cytoplasmic pools of activated eneidynes by quenching reactive benzyne radicals through C-H abstraction from a glycine residue in the active site binding calyx resulting in CalC cleavage [81,82]. It is unclear if antibiotic sequestration by reversible or self-sacrificing SBPs represents a clinical antibiotic resistance threat given that the large doses of antibiotics used in clinical settings are likely to outcompete sequestration as a viable resistance strategy.

No matter the evolutionary trajectory for emergence of antibiotic inactivating enzymes, ultimately, the antibiotic scaffold determines the modification site and type of enzymatic inactivation that will be effective for a given antibiotic class (Table 1) [11,14,17,22,31,50–53,57,59,60,65,69,71,72,74,76-,80–82,84–130]. Highly conserved functional groups and reactive "soft spots" in the scaffold (e.g., strained beta-lactam rings) are prime targets for inactivating resistance enzymes. Resistance can only be achieved if the chemical modification is irreversible on a biological time scale and if the modification blocks access and/or binding to the target. The most dangerous antibiotic inactivating enzymes completely "destroy" the antibiotic scaffold (e.g., beta-lactamases and tetracycline destructases), possess broad substrate coverage across the entire antibiotic class, and are expressed in response to the antibiotic challenge. Presumably, no antibiotic, even the famed teixobactin claimed to be free of "detectable resistance" (see previous discussion), can fully evade emergence of inactivating enzymes [47]. Recently, Li and coworkers [85] discovered a D-stereospecific peptidase associated with NRPS BGCs that can confer pan resistance to antibacterial NRPs containing D-amino acids. Specifically, two D-stereospecific peptidases, BogQ and TriF, from the bogorol and tridecaptin BGCs, respectively, were shown to confer self-protection to the producing microbes via enzymatic hydrolysis of the corresponding NRPs *in vitro*. Surprisingly, BogQ was also able to hydrolyze the non-native substrate bacitracin and confer resistance to bacitracin in strains expressing BogQ. While a truncated teixobactin peptide was not a very good substrate for BogQ, this type of peptidase could emerge as a teixobactin-inactivating enzyme. Directed evolution of BogQ with functional selections against teixobactin might be a good way to anticipate gain-of-function mutations in D-stereospecific peptidases leading to teixobactin resistance.

Prospecting for Resistance

The emergence of environmental antibiotic resistance mechanisms in human pathogens through horizontal gene transfer is now recognized as a major route for dissemination of ARGs in the community and clinical settings (Fig. 7) [28,30]. Monitoring for antibiotic resistance using functional screens (e.g., antibacterial susceptibility assays on clinical isolates) in hospitals is a long-standing clinical practice [131]. New advancements in next-generation DNA sequencing and functional metagenomics have expanded the capacity to prospect for resistance in environments where microbial cultivation is challenging [28,132]. Functional metagenomics has proved useful in revealing new members and new families of ARGs providing a refined view of sequence diversity and environmental distribution of a given ARG class [22]. Historically, resistance among antibiotic producers and heterologous hosts has been used advantageously by pharmaceutical companies to screen for new antimicrobial agents in natural product extracts at the beginning of the antibiotic discovery process. Leveraging modern advancements in genetics and analytical instrumentation revisiting these fruitful discovery paradigms are turning up new molecules and revealing associated biosynthesis and resistance pathways.

Functional metagenomic screens for ARGs

In a seminal study, Wright and coworkers [39,133] demonstrated that the equilibrium pool of ARGs in soil actinomycetes is rich. It was no surprise to discover that environmental resistance to natural product-derived antibiotics such as macrolides is more common than resistance to fully synthetic antibiotics such as linezolid. Pioneering work by Allen and coworkers [134] revealed the diversity, distribution, and origins of beta-lactamase resistance genes in Alaskan soil and validated soil environments as a reservoir of resistance even in the absence of anthropogenic activity. The Dantas laboratory expanded on this principle in the search for environmental ARGs by broadly applying functional metagenomics using environmental DNA

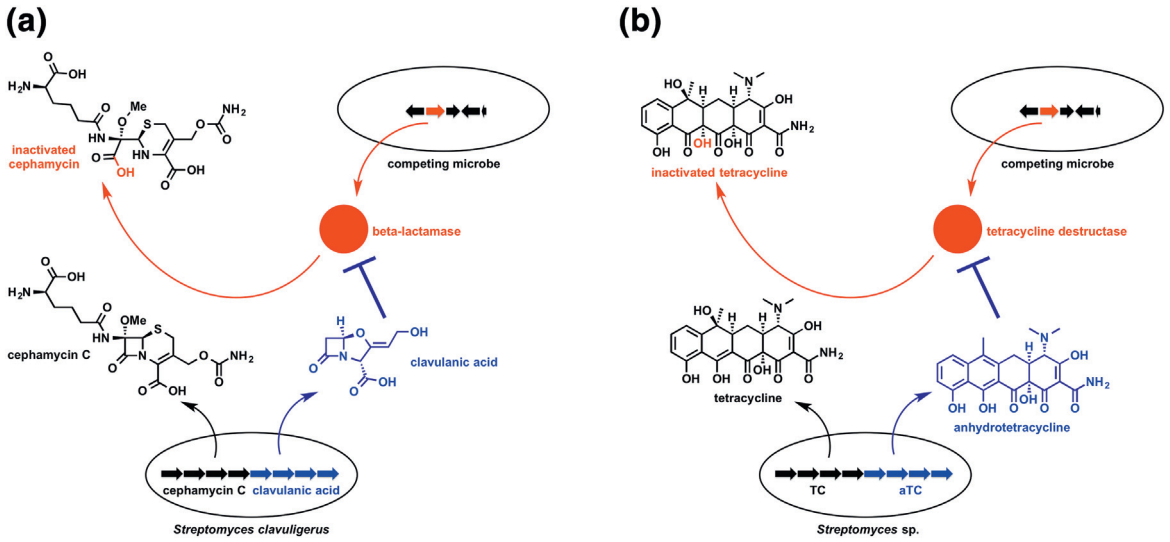

Fig. 8. Co-production of antibiotic (black) and adjuvant (blue) to overcome resistance during microbial competition. (a) Transcription of the beta-lactam "super" BGC in *S. clavuligerus* results in co-production of cephamycin C and clavulanic acid, a beta-lactam antibiotic/beta-lactamase inhibitor combination that rescues beta-lactam antibacterial activity against competing microbes expressing beta-lactamase resistance enzymes. (b) Similarly, co-production of anhydrotetracycline (aTC), a tetracycline destructase inhibitor, and tetracycline (TC), a ribosome inhibitor, might provide a competitive advantage against microbes expressing tetracycline destructase resistance enzymes.

samples from diverse environments to screen for antibiotic resistance against all major classes of antibiotics in an *E. coli* heterologous host [135]. By carefully screening soil and human gut metagenomes, the connection between ARGs in soil microbes, human commensals, and pathogens was solidified. Functional screens can reveal ARGs associated with all mechanistic types of antibiotic resistance, including enzymatic inactivation.

In the case of antibiotic inactivating enzymes, functional metagenomics combined with *in vitro* reconstitution has played a key role in validating enzyme function connected to antibiotic resistance [28,132]. The flavin-dependent tetracycline inactivating FMOs highlighted throughout this review were discovered during functional metagenomic analysis of soil and human gut microbiomes (Fig. 4) [22,24]. Homologous FMOs that inactivate rifamycin antibiotics via similar oxidative degradation mechanisms have been the focus of recent study by Liu *et al.* [125,136] and Koteva *et al.* [126]. The pool of rifamycin FMOs could presumably be expanded using functional metagenomic screens as done for the related tetracycline FMOs. Functional screens have also led to the discovery of previously overlooked antibiotic inactivating enzymes such as chloramphenicol nitro reductases [128,129]. Related approaches have even turned up novel antibiotic degradation mechanisms including "antibiotic eater" strains that provide an expanded view of the natural roles for antibiotics beyond bacterial growth inhibition [95,108]. Antibiotic catabolism in environmental microbes could turn out to be a rich source of antibiotic inactivating enzymes [137]. Expanding known ARGs and enriching the sequence pool for a given ARG class is the first step in gaining predictive capacity for resistance based solely on genome sequencing. Building predictive sequence similarity networks for ARGs will be an important tool for identifying emerging clinically relevant ARGs in human pathogens. Even with increasingly diverse genomic and metagenomic sequence databases, it is still difficult to predict functional ARGs from sequence alone. A recent study by Ruppe and coworkers [138] revealed that coupling functional metagenomics with three-dimensional protein structure prediction through pairwise comparative modeling, so called "homology comparative modeling," can refine the pool of predicted ARGs and provide new candidate antibiotic inactivating enzymes that would otherwise remain overlooked by direct sequence comparision.

Computational approaches to predicting resistance

New innovations in computational methods to predict function based on sequence and structural identity will play an important role alongside functional screens in future campaigns to expand ARG predictive capacity [28,132,138]. Ancestral reconstruction of ARGs present in the clinic will connect gain-of-function mutants in natural environments and clinical settings where the evolutionary

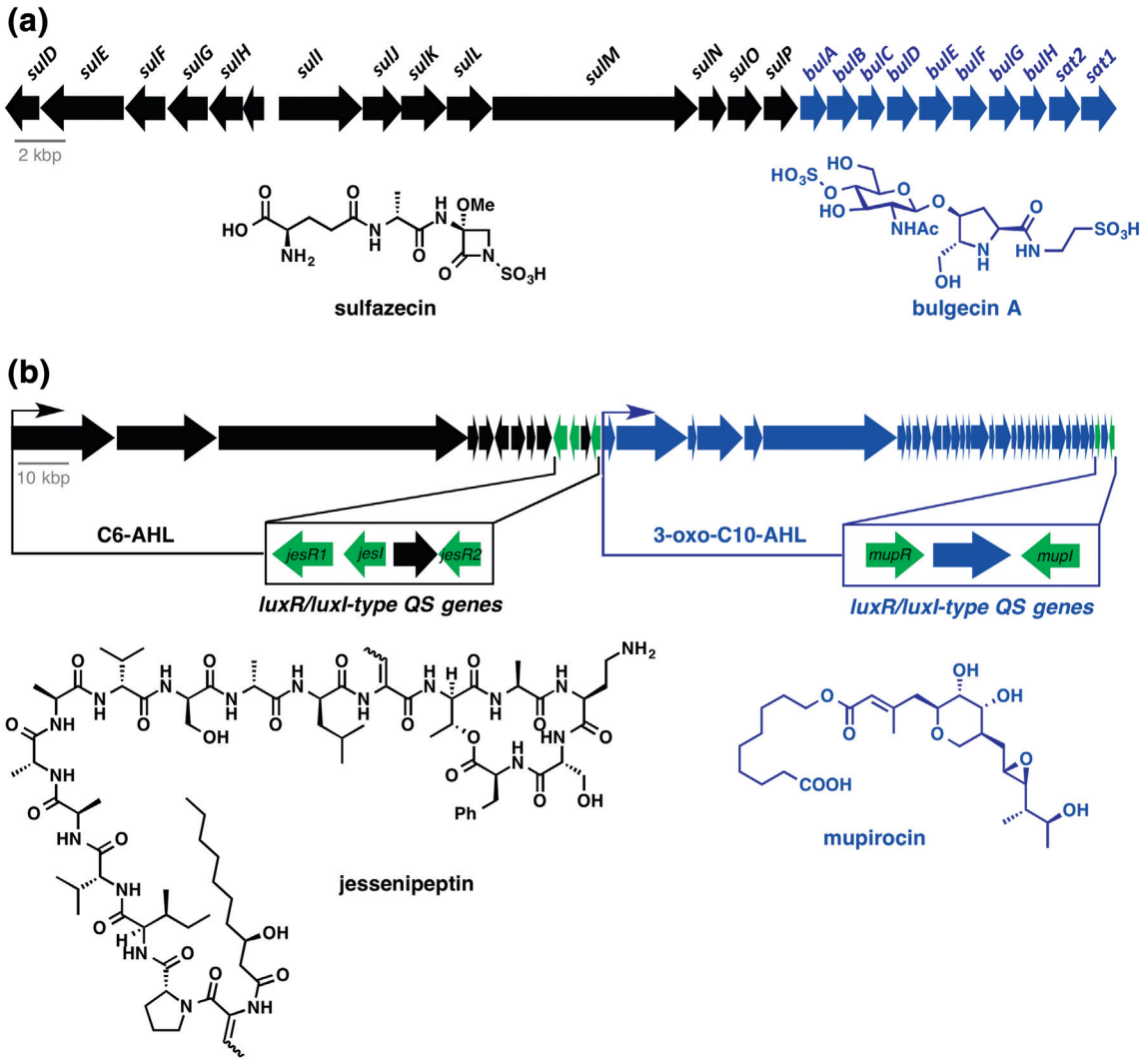

Fig. 9. Co-production of synergistic antibiotic combinations to improve potency and limit resistance development. (a) Sulfazecin and bulgecin BGCs in *P. acidophila* ATCC 31363 are arranged as a "super cluster." (b) Mupirocin and jessenpeptin BGCs in amoeba-associated *Pseudomonas* sp. QS1027 are co-regulated by LuxR/LuxI-type quorum sensing (QS) systems. Genes are color coded blue or black to match biosynthetic products (green genes are regulatory).

connection is not necessarily mutually exclusive [71,135]. Purely computational approaches for predicting protein evolution are hampered by molecular ensembles and statistical thermodynamics [139]. The use of Markov state models is gaining traction to explore cryptic functional states of proteins, including TEM beta-lactamases, that can provide positive and negative allosteric modulators when coupled to small molecule screens [140,141]. Directed evolution and deep mutational scanning has proven to recreate clinically relevant gain-of-function mutations in TEM beta-lactamases. In a seminal study by Orencia and coworkers [142], TEM-1 was subjected to directed evolution in hypermutator *E. coli*, which recreated three mutations (E104K/M182T/G238S) found in the clinical beta-lactamase TEM-52 that improve stability and impart activity against third-generation cephalosporins such as cephotaxime. Similar approaches have been applied to aminoglycoside and macrolide inactivating enzymes, suggesting that computational methods will need to be supplemented with functional screens to provide useful information on the catalytic efficiency, cellular abundance, stability, and evolutionary trajectory of antibiotic resistance enzymes in a given pathogen [71,77].

Resistance-guided antibiotic discovery

The link between antibiotic biosynthesis and resistance is playing an increasingly important role in prospecting for antibiotic resistance and next-

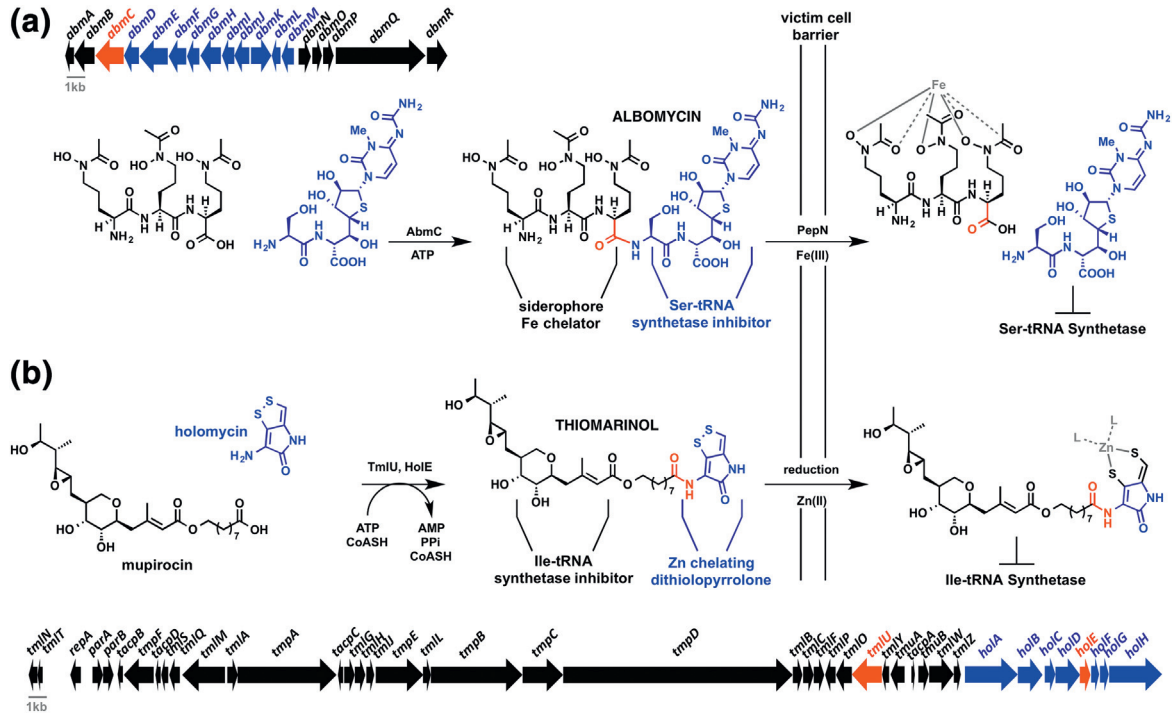

Fig. 10. Biosynthesis and activation of hybrid antibiotics to improve potency, engage multiple biological targets, and limit resistance development. (a) Albomycin BGC from *Streptomyces* sp. ATCC 700974 encodes an amide synthetase AbmC that presumably ligates the siderophore and Ser-tRNA synthetase inhibitor components to form the hybrid sideromycin antibiotic. (b) Thiomarinol BGC from *Pseudoalteromonas* spp. SANK73390 encodes for a CoA-ligase TmlU and acyl transferase HolE that ligates the mupirocin and holomycin components to form the hybrid thiomarinol antibiotic. Genes are color coded to match biosynthetic products.

generation antibiotics. Microbial antibiotic BGCs are a long-term repository for ARGs in diverse environments [34,39,41,133,135]. Resistance-guided BGC mining has turned up several new antibiotics in recent years and has helped to expand the structural pool of known antibiotic classes. Thaker and coworkers [143] reported the first successful demonstration of this method in 2013. By screening a small library of 1000 actinomyces against vancomcyin a 4% resistance hit rate was achieved that correlated with expression of the canonical *vanHAX* operon for glycopeptide resistance. Genome sequencing guided by co-clustering of the *vanHAX* operon with glycopeptide BGCs led to the discovery of pekiskomycin, a scaffold novel glycopeptide. Resistance-guided screening has proved even more useful in recent years where the number of genome-sequenced strains of antibiotic producers is on the rise [144]. Advancements in computational methods and smart screens that combine structure/resistance prediction with functional screens can greatly enhance the rate of antibiotic dereplication to reveal novel antibiotic structures and adjuvants [145,146]. Co-clustering of a duplicate DNA sliding clamp variant (GriR) with a BGC in *Streptomyces griseus* led to the discovery of a new NRP antibiotic, griselimycin, and confirmation of the DNA polymerase sliding clamp domain (DnaN) as the cellular target [147]. Heterologous expression of GriR in *Streptomyces coelicolor* conferred griselimycin resistance. Co-crystallization of griselmycin with the *Mycobacterium smegmatis* DNA sliding clamp dimer revealed a novel binding mode in adjacent hydrophobic pockets created by an interdomain protein–protein interaction that is stabilized by the macrocyclic NRP core and proline–valine tail of griselimycin, respectively. Griselimycin and the DNA clamp represent a novel antibiotic/target pair with promising anti-TB activity. Future resistance-guided screens against griselimycin paired with detailed analysis of the biosynthetic assembly will likely turn up novel structural analogs [148]. More recently, Chang and coworkers [80] have connected three proteins, TnmS1–S3, with anthraquinone-fused enediyne self-resistance in producers. The TnmS1–S3 proteins are beta-barrel homodimers that bind the enediynes with nanomolar affinity providing resistance via sequestration. The genes encoding these resistance proteins are conserved in producer BGCs and widespread in diverse environments including the human microbiome. Enrichment of the human microbiome with ARGs creates a selection-ready environment for resistance upon clinical antibiotic use.

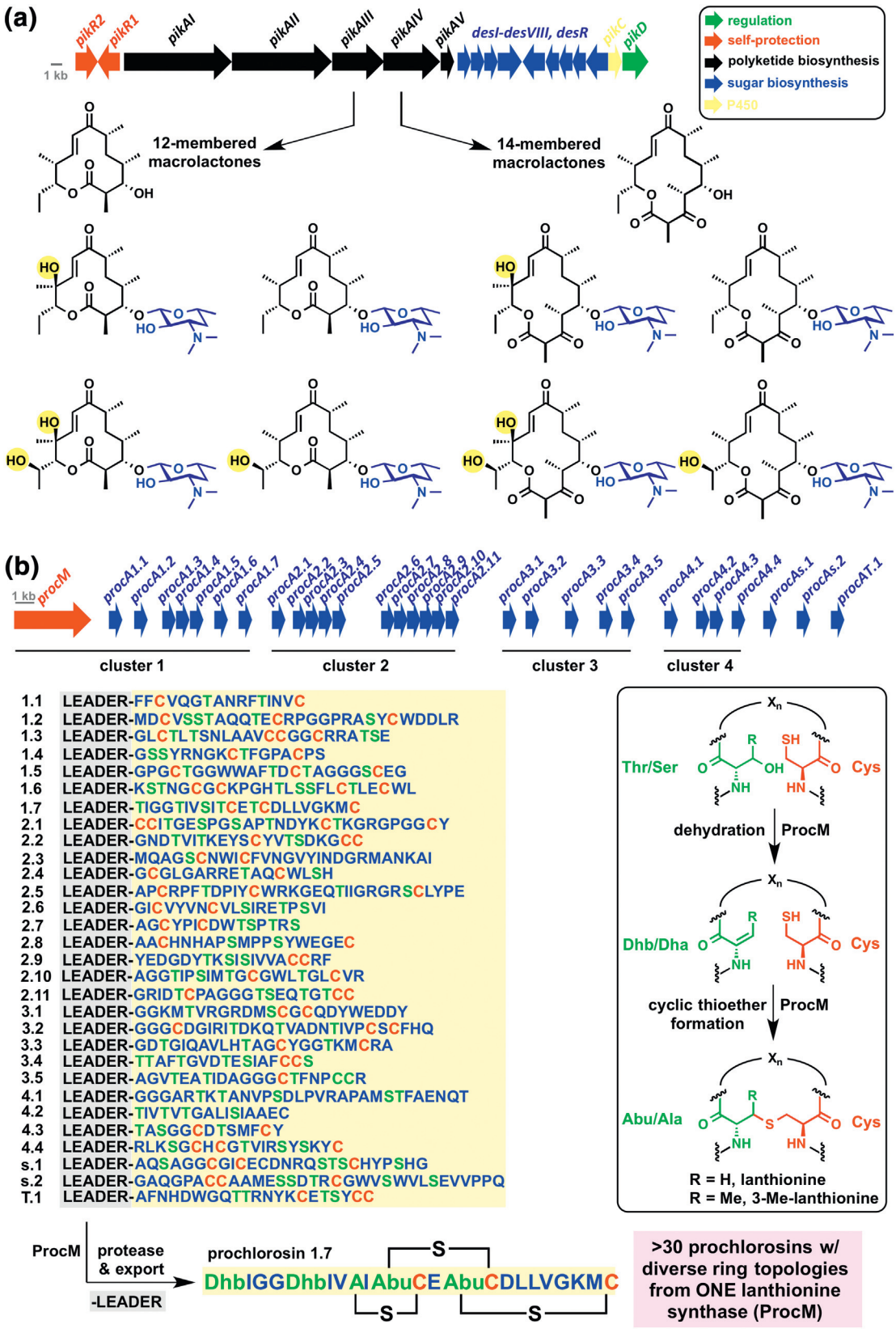

Fig. 11 (*legend on next page*)

Philmus and coworkers recently highlighted a nice selection of ARG–BGC genome mining efforts including blasticidin (BlsJ; efflux pump) [149], andrimid (AdmT; beta-subunit of the target acetyl coA carboxylase) [150], mycophenolic acid (MpaF; resistant homolog of the target inosine-5′-monophosphate dehydrogenase) [151], platensimycin/platencin (PtmP3; resistant homolog of the target beta-ketoacyl-acyl carrier protein synthase III) [152], indolmycin (IndO; resistant homolog of the target Trp-tRNA synthetase) [153,154], and geldanamycin (HtpG; resistant homolog of the target heat shock protein 90) [155,156]. New examples of ARG–-BGC mining continue to emerge including the recent discovery by Petronikolou *et al*. [157] of a resistant threonine synthase homolog ThrC in *Bacillus subtilis* ATCC 6633, a natural producer of the potent phosphono-oligopeptide threonine synthase inhibitor rhizocticin. In this case, *in vitro* reconstitution of sensitive and resistant ThrC homologs from *B. subtilis* provided mechanistic insight into the mode of threonine synthase inhibition that can guide the design of improved phosphono-oligopeptide inhibitors that regain activity against current insensitive ThrC targets. Resistance-guided genome mining combined with advancements in high-throughput microbial genome sequencing and mass spectrometry provides new opportunities in natural product discovery [145,146,158]. The targeted mining for ARGs embedded in BGCs will help to distinguish antibacterial agents from natural products with alternate functions. Furthermore, heterologous expression of BGC-derived ARGs can be used to screen for new producers and identify structural relatives to expand the pool of a given natural product class. Utilization of isogenic panels of bacterial strains expressing clinical ARGs has already proven useful as an antibiotic discovery strategy and a way to optimize antibiotic structures to evade resistance [8,159].

Staying Ahead of Resistance

Antibiotic resistance is something that should be considered at the earliest stages of antibiotic development. Resistance should be managed, not avoided, through pharmaceutical optimization and responsible stewardship following deployment into medical practice [160]. A pro-active resistance-management plan should be incorporated into the development of all new antibiotics to extend the clinically useful lifetime and allow for sustainable introduction of future scaffold generations. While nature is the origin of antibiotic resistance, it also holds the solutions. Nature has provided a plethora of novel antibiotics acting on unexploited biological targets that still await pre-clinical development [29]. These molecules represent great starting points for new antibiotic development programs where resistance has not saturated the target and scaffold iteration has not saturated the market. Rediscovery of new variants of established clinical antibiotics reveals scaffold modifications that might improve potency, evade established resistance mechanisms, or provide a useful pro-drug delivery strategy. In addition, genome mining is proving to reveal that antibiotic BGCs are often grouped into “super clusters” that enable co-production of synergistic antibiotics or hybrid antibiotics with enhanced bioactivity.

Synergistic antibiotic combinations

The first documented example of a biosynthetic “super cluster” is the cephamycin–clavulanate BGC in *Streptomyces clavuligerus* and related *Streptomycetes* [161,162]. The cephamycin and clavulanate operons are co-regulated by the *ccaR* gene in the cephamycin BGC [163]. CcaR is a *Streptomyces* antibiotic regulatory protein (SARP) that acts as a positive regulator of the cephamycin–clavulanate “super cluster.” CcaR over-expression leads to increased production of cephamycin and clavulanate [164]. This co-production of two beta-lactams, cephamycin and clavulanate, is beneficial given the specific bioactivity of each molecule (Fig. 8a). Cephamycin is a cephalosporin antibiotic that inhibits bacterial penicillin binding proteins and is susceptible to some classes of beta-lactamase enzymes. Clavulanic acid is a potent inhibitor of many beta-lactamases and rescues beta-lactam antibiotic activity against competing microbes expressing beta-lactamase enzymes [165]. It should also be noted that *S. clavuligerus* secretes a beta-lactamase inhibitory protein (BLIP) in addition to clavulanic acid [166,167]. Structural details of proteinaceous BLIPs in complex with inhibited beta-lactamases might offer a starting point for designing new small molecule inhibitors [168–170]. Clavulanic acid has been developed as a combination product with clinical beta-lactam antibiotics,

Fig. 11. Biosynthetic promiscuity as a source of antibiotic cocktails. (a) Pikromycin biosynthesis in *S. venezuelae* produces a pool of 12- and 14-membered macrolides with varying degrees of side chain oxidation. Genes are color coded as defined in the legend where the color of biosynthetic genes match the structural fragment derived from catalysis by enzyme gene products. (b) Prochlorosin biosynthesis in *Prochlorococcus* MIT9313 generates a library of structurally diverse lantipeptides with unique ring topologies from genetically encoded precursor peptides and a single lanthionine synthetase ProcM with low substrate selectivity. The *procM* gene is shown in red while all genes encoding product precursor peptides are shown in blue.

including the penem antibiotic amoxicillin forming a combination antibiotic therapy known as Augmentin™ [171].

There is a key lesson that may apply to other natural products where co-expression of multiple biosynthetic products provides synergistic bioactivity. For example, anhydrotetracycline was recently reported to be a potent inhibitor of tetracycline destructase resistance enzymes isolated from environmental soil microbes [24,25]. Anhydrotetracycline is also the biosynthetic precursor to tetracycline with both metabolites accumulating in the extracellular space of the producing microbe (Fig. 5b) [32,33]. It could be that co-production of anhydrotetracycline and tetracycline is "intentional" and provides a growth advantage against competing microbes expressing tetracycline destructase enzymes (Fig. 8b) [14]. Genome mining and smart screens can also reveal antibiotic adjuvants whether or not co-clustering is at play [145]. Recently, King and coworkers [172] used such a method to identify aspergillomarasmine A, a potent inhibitor of metallo-beta-lactamases including NDM-1 for which there is currently no clinically useful inhibitor. Revisiting natural product discovery with an angle toward antibiotic combination discovery is needed to enrich the pool of antibiotic potentiating agents to validate combination therapies as a sustainable option for managing resistance [173].

A more recent example of a biosynthetic "super cluster" encoding co-production of sulfazecin and bulgecin was discovered in *Paraburkholderia acidophila* ATCC 31363 and *Burkholderia ubonensis* ATCC 31433 (Fig. 9a) [174]. Sulfazecin is an *N*-sulfated monobactam inhibitor of the transpeptidase domain of bacterial penicillin binding proteins, while bulgecin is a 2-(hydroxymethyl)-3-(*O*-(*N*-acetyl-D-glucosamine-4-sulfate))-pyrrolidine inhibitor of lytic transglycosylases MltD, MltG, and Slt [175]. Consequently, co-production of sulfazecin and bulgecin results in simultaneous inhibition of peptidoglycan peptidyl crosslinking and glycan chain hydrolysis, respectively, worsening the effects of the cell wall repair futile cycle and leading to rapid cell lysis [176]. The bactericidal effects of sulfazecin and bulgecin are synergistic, and bulgecin has been shown to potentiate the antibacterial activity of clinical beta-lactam antibiotics including third-generation cephalosporin ceftazidime and meropenem. Further investigation of bulgecin and beta-lactams as combination therapeutics is merited. The bulgecin/sulfazecin "super cluster" is a reminder that stand alone antibiotic BGCs should be revisited to see if flanking genomic operons harbor BGCs for synergistic molecules. Recently, it was realized that all producers of rapamycin, a well-known immunosuppressant with antifungal activity, also produce a group of actinoplanic acids that provide a synergistic antifungal effect via inhibition of farnesyltransferases [177]. The rapamycin and actinoplanic acid BGCs are organized as "super clusters" in producing microbes. Prior to this discovery, combinations of rapamycin and farnesyltransferase inhibitors were found to have synergistic effects against several types of mammalian cancers and are currently under clinical investigation [178].

Another example of co-antibiotic production was recently discovered in *Pseudomonas* sp. QS1027, an amoeba-associated strain isolated from the fruiting bodies of *Dictyostelium discoideum* in soil [179]. *Pseudomonas* sp. QS1027 was found to produce the polyketide antibiotic mupirocin, a clinically useful inhibitor of Ile-tRNA synthetase marketed as Bactroban®, along with a cooperatively excreted cyclic lipopeptide, jessenipeptin, produced via an NRPS pathway (Fig. 9b). The jessenipeptin and mupirocin BGCs are organized in a "super cluster" and both sub-BGCs contain a dedicated *luxR*/*luxI*-type pair of quorum sensing (QS) genes. Production of mupirocin and jessenipeptin is stimulated by unique acyl homoserine lactones (AHLs) (C6-AHL activates transcription of the jessenipeptin BGC; 3-oxo-C10-AHL activates transcription of the mupricon BGC) indicating a role for quorum sensing in the cooperative biological activity of the antibiotic pair. Co-production of mupirocin and jessenipeptin did not play a role in the immunity of strain QS1027 to *D. discoideum* grazing, although jessenipeptin, not mupirocin, did show potent amoebicidal activity (IC_{50} = 4 μg/mL). However, the antibiotics were synergistic in the killing of gram-positive bacteria including *B. subtilis* and methicillin-resistant *Staphylococcus aureus*. The target of jessenipeptin is unknown, but the synergistic relationship with mupirocin, a Ile-tRNA synthetase inhibitor, suggests membrane depolarization as a potential mechanism of action that would enhance cell permeability of mupirocin allowing for greater cytoplasmic accumulation. Mupirocin–jessenipeptin synergy points to a role for co-biosynthetic regulation of the antibiotic pair by unique quorum sensing agents that might contribute to the adaptation of certain pseudomonads to coexist with predatory amoebas. In the broader sense, this example should encourage the study of microbial interactions in ecology to better understand the plethora of "silent" antibiotic BGCs found in microbial producers that reserve antibiotic deployment for highly specialized scenarios [180].

Hybrid antibiotics

An alternative evolutionary approach to antibiotic "super clusters" resulting in co-production of distinct antibiotic entities is the merging of BGCs through biosynthetic enzymes that catalyze the covalent ligation of multiple antibiotic scaffolds creating multifunctional hybrid antibiotics [181–183]. Here the term "hybrid antibiotic" is used to define the joining

of two different antibiotic moieties via a biosynthetic pathway present in a single organism, as opposed to the traditional use of the term "hybrid" in plant and animal breeding where the product results from the combination of genes from two disparate strains or species. There are, however, some parallels between the two uses of the term since hybrid antibiotic biosynthetic operons in a single microbe might arise from a horizontal gene transfer event between two disparate microbes each harboring biosynthetic operons for individual antibiotic moieties that end up in the hybrid scaffold. Hybrid antibiotics can take advantage of expanded target engagement and leverage access to membrane transport pathways provided by the merging of two antibiotic scaffolds.

Classic examples of hybrid antibiotics are the naturally occurring sideromycins (siderophore-antibiotic conjugates), which achieve energy-dependent receptor-mediated import across the cell envelope through iron transport pathways to access periplasmic and cytoplasmic targets of the antibiotic attached to the siderophore delivery vector (Fig. 10a) [184]. In a sense, the sideromycins are similar to dipeptide prodrugs such as tabtoxin that were discussed earlier (Fig. 3), where the siderophore component facilitates cell entry in a manner similar to the dipeptide prodrug [57,58,91]. Since siderophore transport is driven by ATP hydrolysis, intracellular concentrations of the antibiotic can reach levels that well exceed extracellular concentrations leading to enhanced potency, often in the nanomolar range [184]. The location and nature of the biological target determines if antibiotic release from the siderophore is required for target engagement [185–187]. In the case of albomycin, a thionucleoside Ser-tRNA synthetase inhibitor is linked to a ferrichrome-like trihydroxamate siderophore through a peptide bond that is cleaved by the cytoplasmic peptidease PepN found in many gram-negative bacteria (Fig. 10a) [188]. PepN deletion mutants are resistant to albomycin reflecting that antibiotic release from the siderophore is critical for antibacterial activity [189]. Resistance can also be achieved via mutations or deletion of transport proteins, indicating that targeted uptake pathways must be required for pathogen virulence [190,191]. Albomycin serves as a model template for hybrid antibiotic biosynthesis where the ferrichrome siderophore is assembled via a dedicated NRPS assembly line and the thionucleoside is produced via an independent series of enzymes encoded in the same BGC [192,193]. An ATP-dependent ligase, AbmC, is predicted to join the siderophore and thionucleoside via a serine linker. A similar convergent strategy was used to assemble the albomycin scaffold via total synthesis opening the opportunity for the production of new analogs for pharmaceutical optimization [194]. It is noteworthy that the albomycin BGC also contains a duplicate copy of Ser-tRNA synthetase, AbmK, that serves a role in self-protection against poisoning of the canonical Ser-tRNA synthetase encoded outside the albomycin BGC [192]. The sideromycin drug delivery approach has gained wide traction in academic and industrial settings as a method for developing pathogen-targeted narrow spectrum antibacterial agents and platforms for pathogen diagnostics, although it should be noted that pre-clinical toxicity and resistance development have hindered clinical study of sideromycins [184,191]. Cefiderocol is the lone example of a siderophore cephalosporin that has shown promise through phase 2 clinical trials and is being pursued as a treatment for MDR gram-negative pathogens such as *Pseudomonas aeruginosa* [195].

A more recent example of biosynthetic hybridity is the antibiotic thiomarinol C produced by *Pseudomonas* spp. SANK73390 [196,197]. Thiomarinol C is composed of pseudomonic acid C, a component of the FDA-approved antibiotic mupirocin, joined to the dithiolopyrrolone antibiotic holomycin (Fig. 10b) [198]. Pseudomonic acid C is an Ile-tRNA synthetase inhibitor, while holomycin has been shown to influence Zn-dependent metabolic pathways and inhibit some Zn-dependent metalloenzymes [199,200]. Thiomarinol C is at least equally as potent an inhibitor of Ile-tRNA synthetase as the parent pseudomonic acid C and benefits from improved cell permeation, improved spectrum of activity, and increased stability [201]. It is unclear if holomycin is cleaved from mupirocin in the cytoplasm, but this does not seem to be required since the intact thiomarinol scaffold remains inhibitory toward the target Ile-tRNA synthetase. Furthermore, the holomycin moiety might potentiate the inhibition of protein synthesis through Ile-tRNA synthetase inhibition via disruption of RNA replication and other Zn-dependent metabolic pathways [199,200]. Thiomarinol biosynthesis is analogous to albomycin biosynthesis in that each antibiotic component is produced separately by genes contained in the same biosynthetic "super cluster" with a final ligation step of the two antibiotic components [196,202,203]. The free carboxylate of pseudomonic acid C is converted to the corresponding coenzyme A thioester by the ATP-dependent ligase TmlU. Acyltransferase HolE catalyzes nucleophilic attack of the thioester carbonyl by the free amino group of holomycin to give free conenzyme A and the amide linked holomycin-pseudomonic acid C conjugate, thiomarinol C, following nucleophilic acyl substitution. The reconstitution of TmlU/HolE could enable the chemoenzymatic production of new thiomarinol inspired hybrid structures to complement existing methods based on chemical synthesis and mutasynthesis [201,203–205]. Hybrid antibiotics offer a potential advantage if one component faces modification by

an inactivating enzymes since the second antibiotic component might remain functionally active. Mining microbial BGCs for hybrid antibiotics is needed to complement current synthetic strategies aimed at synthesizing multifunctional hybrid antibiotics that overcome established resistance mechanisms [181–183]. New advancements in synthetic biology [206,207], protein engineering [208], chemoenzymatic synthesis [209], and heterologous natural product expression [210] are needed to advance the study and production of new hybrid antibiotics in engineered microbes.

Biosynthetic promiscuity

According to the *screening hypothesis*, promiscuity in natural product biosynthesis provides an evolutionary advantage [211]. That is, organisms that produce a larger number of antibiotics, at the potential cost of less potency, have a greater chance of making beneficial compounds for a more diverse set of evolutionary scenarios. Biosynthetic promiscuity can be obtained in several ways including enzyme substrate plasticity [212], post-biosynthetic tailoring reactions [213], release of shunt biosynthetic products [214–216], and even non-enzymatic post-biosynthetic scaffold rearrangements [217–220]. Macrolides represent a classic example in biosynthetic diversity where control of macrocycle ring size, degree of framework oxidation, and post-polyketide synthase glycodiversificiation results in production of a structurally diverse pool of macrolides (Fig. 11a) [78,216]. The pikromycin polyketide synthase (PKS) assembly line in *Streptomyces venezuelae* provides at least six structurally distinct macrolide antibiotics. The PKS is composed of six modules (PikAI–IV) and a stand-alone type II thioesterase (PikAV) that acts *in trans* to release and cyclize the penultimate thioester precursor to the macrolide cores. Remarkably, it appears as though PikAV can cleave and cyclize thioesters from both module 5 and module 6 resulting in the formation of 12- and 14-membered macrolactones, respectively, that serve as substrates for a downstream P450 oxidase PikC and a glycosyltransferase DesVII that generate the final cocktail of pikromycin protein synthesis inhibitors with a high degree of substrate plasticity. In the laboratory, the substrate plasticity of the macrolide post-PKS modification enzymes has proved useful in the chemoenzymatic diversification of macrolides through late-stage enzymatic oxidations and glycorandomization [213,221,222]. While directed evolution approaches for tailoring enzyme optimization typically turn up highly specific enzymes, natural evolution seems to favor tailoring enzymes with a high degree of substrate plasticity [223]. Presumably, producing a pool of macrolides increases the odds of overcoming resistance by the armament of environmental macrolide inactivating enzymes by supplying decoy substrates, non-substrates, and possibly inhibitors.

More recently, the explosion of research in the area of ribosomally synthesized post-translationally modified peptide (RiPP) family of antibiotics has revealed some of the biosynthetic secrets that lead to immense structural diversification of small peptides derived from proteinogenic amino acid building blocks [224]. Surprisingly, the enzymes that perform the post-translational modifications (PTMs) following ribosomal generation of RiPP precursor peptides display a large degree of substrate plasticity that is tamed by inclusion of a traceless leader peptide that guides substrate peptides to enzymes responsible for installing PTMs. One PTM common to the lanthipeptide subclass of RiPPs is the conversion of a Ser or Thr to the corresponding dehydroalanine (Dha) or dehydrobutyrine (Dhb) residue, respectively, via *O*-glutamylation or *O*-phosphorylation followed by dehydration with loss of glutamate or phosphate, respectively (Fig. 11b). Nearby Cys residues can add as nucleophiles in a 1,4-conjugate addition (Michael reaction) to the newly formed Dha or Dhb residues followed by α-protonation of the resulting enolate to give a cyclic thioether known as a lanthionine or 3-methylanthionine residue, respectively. Bifunctional lanthipeptide synthetases (LanM) that catalyze tandem dehydrative conjugate additions to form lanthionine residues in lantipeptide antibiotics show great substrate plasticity and result in the formation of structurally diverse cocktails of lanthipeptides with varying degrees of macrocyclizations and dehydrations [225]. In nisin biosynthesis, NisC catalyzes the formation of all five cyclic ethers in the lipid-II sequestering lantipeptide scaffold [226]. The prochlorosin lantipeptides produced by the cyanobacterium *Prochlorococcus* MIT9313 represent a more extreme example of combinatorial diversity-generating biosynthesis (Fig. 11b) [227]. The genome of *Prochlorococcus* MIT9313 encodes at least 30 ProcA lantipeptide pre-peptides and only one bifunctional lantipeptide synthase, ProcM [228]. The ProcA peptides (1.1–1.7; 2.1–2.11; 3.1–3.5; 4.1–4.4, s.1, s.2, T.1) share >60% sequence similarity in the leader peptide and <30% sequence similarity in the antibiotic pre-peptide ranging from 12 to 32 amino acids [229]. Nearly all possible Thr/Ser dehydrations and Cys cyclic thioether formations of the peptide cocktail are formed enzymatically by ProcM resulting in a staggering array of ring topologies for the kinetically controlled pool of >30 lantipeptides excreted by *Prochlorococcus* MIT9313 [230,231]. This level of biosynthetic flexibility has inspired several approaches to building DNA-encoded libraries of RiPP antibiotics using codon randomization and even phage display in yeast [232–234]. While cell-based approaches to RiPP diversification will presumably eliminate production of compounds that are toxic to the host, the ease of producing large compound libraries via these systems

should enable sufficient chemical diversity to provide hits in a wide range of therapeutic areas including "undruggable" protein–protein interactions [235]. Advancements in the *in vitro* reconstitution of RiPP biosynthetic enzymes have also enabled chemoenzymatic approaches to certain members of the RiPP antibiotic family including pyridine-containing thiopeptides and lantipeptides [209]. RiPP antibiotics show a wide range of biological activity, and many show promise as antimicrobial and anticancer agents [236]. Perhaps pursuing defined cocktails of RiPPs for desired therapeutic applications will provide a higher hit rate for lead optimization and outpace resistance development. The topic of "diversity-generating biosynthesis" was recently taken up by Gu and Schmidt [237], and the argument is made that enzyme "promiscuity" seems to be the rule rather than the exception for certain pharmacophores, including lanthionine units, where scaffold diversification provides a competitive advantage against resistance development consistent with the natural product "screening hypothesis" first proposed by Firn and Jones [211].

Final Thoughts

Turning to nature's therapeutic logic and resistance management plans is a smart way to supplement and inspire new directions to overcome antibiotic resistance in the clinic [29,238]. This philosophy has proved fruitful in providing the first wave of antibiotic scaffolds that has sustained 80 years of clinical success. Today new antibiotic drugs, including multiple iterations of beta-lactam antibiotic and beta-lactamase inhibitor combinations, derived from natural products continue to provide essential cures for bacterial infections. The slow pace of new antibiotic classes entering the clinic and increasing rates of resistance against new and established antibiotic drugs demands constant nurturing of the antibiotic discovery pipeline [239]. Scaffold iterations are no longer keeping pace with antibiotic resistance [240]. There are many lessons still to learn from the balance of antibiotic resistance and susceptibility in natural environments that can inspire new therapeutic strategies and methods for managing antibiotic resistance. In natural environments, resistance is at equilibrium and microbial populations welcome all phenotypes including antibiotic producers, sacrificial lambs, cheaters, and police [241,242]. While resistance has reached equilibrium in natural environments, resistance in hospitals has tipped in favor of MDR pathogens. While humans did not create antibiotic resistance, we have amplified resistance in certain settings by our practice of antibiotic consumption that is now trickling into the general population [36,37].

Chemical synthesis [243], traditional medicinal chemistry [236], and advances in drug delivery [244] will always serve as a pillar of antibiotic development. It is time again to turn to natural products in the genomics and metabolomics era to provide the next wave of molecules that will sustain decades of infectious disease treatment. Synergistic combination therapies will play an important role in the future of antibiotic therapeutics to both overcome established resistance and delay the emergence of new resistance mechanisms. Mining microbial genomes for "super" and "hybrid" BGCs encoding the production of synergistic antibiotics promises to deliver new lead antibiotic combinations and fuel the development of non-traditional therapeutic strategies. As some molecules are synergistic and others antagonistic, the timing of biosynthetic production is key with transcriptional regulation controlling the molecular pool in response to external signals (e.g., quorum sensing). An improved understanding of this antibiotic regulation in mixed microbial populations will help guide the development and use of antibiotics in the era of harnessing the human microbiome [245,246]. New drug delivery strategies and modification of existing antibiotic scaffolds to favor intracellular accumulation in target bacteria merit continued investigation inspired by natural compounds that efficiently cross the bacterial cell envelope [49]. Advancements in measuring cellular accumulation of antibiotics and exploration of non-traditional antibiotic targets including virulence factors are welcomed additions to antibiotic discovery programs. New smart screens including chemical genetic assays for synthetically lethal gene targets and resistance-guided antibiotic discovery have promise to reveal new antibacterial natural products [159,247]. Recent efforts to improve microbial cultivation [248], activate silent antibiotic BGCs [245,246], and expand metagenomic capture of BGCs expressed in heterologous hosts have turned up new antibacterial leads [249].

The connection between environmental and clinical resistance now seems undeniable. It remains challenging to distinguish between producer self-protection and authentic antibiotic resistance. It is even more difficult to determine if emerging resistance mechanisms from natural environments will have clinical relevance. Based on the short history of antibiotic use, the appearance of environmental resistance in human pathogens is a safe bet if the associated natural antibiotic is widely deployed. Prospecting for emerging resistance and anticipating clinically relevant ARGs using computational methods and functional screens is important to develop resistance management plans for established antibiotics and new lead compounds. Proactive development of new diagnostic agents for specific resistance mechanisms and MDR pathogens along with development of adjuvants for overcoming potential

resistance enzymes should be implemented early in the preclinical development of antibiotics.

In the long run, antibiotic stewardship will probably impact the dissemination of resistance more than any other factor [160]. Our use of antibiotics in medical, agricultural, and related venues should be reevaluated to assess the long-term effects of resistance development on human health, national security, and the economy. For example, the current standard of care for pre-mature infants is administration of antibiotics in neonatal intensive care units [250]. The lack of statistical correlation to increased survival rate combined with concerns about disrupting the developing infant microbiome with an oversized pool of ARGs suggests that long-term consequences of antibiotic use in infants should be investigated [251]. This could be a scenario where prophylactic antibiotic use does more harm than good. A second example worth mentioning is the recent approval by the US EPA to allow the spraying of orange orchards in the state of Florida with the antibiotics streptomycin and oxytetracycline to control citrus greening, a bacterial disease common to Florida citrus trees [252]. This practice could result in the release of 440,000 kilograms of the antibiotics into the soil—an unprecedented scale. Kishony and coworkers [35,253,254] have shown that tetracycline and associated degradation products can dramatically influence microbial population dynamics. It is unclear how large-scale deployment of oxytetracycline and streptomycin will influence the soil and plant microbiomes in the exposure zone, although it is reasonable to hypothesize that tetracycline and aminoglycoside ARGs should increase under selective pressure. These enriched pools of ARGs could compromise the efficacy of newly approved lifesaving aminoglycoside and tetracycline drugs including plazomicin and eravacycline, respectively (Fig. 1) [5,6,9]. The short-sided economic benefits of agricultural antibiotic use are clear, but the potential for antibiotic resistance dissemination might have unintended economic costs related to infectious disease control. In addition, it is unclear if extended large-scale antibiotic use on crops will provide sustainable management of crop disease given that target pathogens will eventually become resistant. This will be a unique opportunity to explore the crossroads of antibiotic resistance and biosynthesis in soil on relevant geographical and time scales.

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Abbreviations used:
AAC *N*-acetyltransferase; APH *O*-phosphotransferase; FMO flavin monooxygenase; MDR multi-drug resistance; BGC biosynthetic gene cluster; ARG antibiotic resistance gene; NRPS non-ribosomal peptide synthetase; GS glutamine synthetase; TBL tabtoxinine-beta-lactam; GNAT Gcn5-related *N*-acetyltransferase; PBP penicillin-binding protein; SBP substrate-binding protein; AHL acyl homoserine lactone; PTM post-translational modification

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