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REVIEW ARTICLE

Targeted antibiotic discovery through biosynthesis-associated resistance determinants: target directed genome mining

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ABSTRACT

Intense competition between microbes in the environment has directed the evolution of antibiotic production in bacteria. Humans have harnessed these natural molecules for medicinal purposes, magnifying them from environmental concentrations to industrial scale. This increased exposure to antibiotics has amplified antibiotic resistance across bacteria, spurring a global antimicrobial crisis and a search for antibiotics with new modes of action. Genetic insights into these antibiotic-producing microbes reveal that they have evolved several resistance strategies to avoid self-toxicity, including product modification, substrate transport and binding, and target duplication or modification. Of these mechanisms, target duplication or modification will be highlighted in this review, as it uniquely links an antibiotic to its mode of action. We will further discuss and propose a strategy to mine microbial genomes for these genes and their associated biosynthetic gene clusters to discover novel antibiotics using target directed genome mining.

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1. Introduction

Microbial natural products (NPs), or secondary metabolites, are of paramount biomedical importance, serving as antibiotics against a variety of pathogenic bacteria. Historically, many of the most important classes of antibiotic scaffolds, including the β-lactams, aminoglycosides, tetracyclines, macrolides, glycopeptides, and lipopeptides, were isolated from microorganisms. Indeed, nearly 60% of US Food and Drug Administration (FDA) approved antibacterial antibiotics for clinical use over the past 30 years (82 out of 140 from January 1 1981 to December 31 2014) have been microbial NPs or derivatives thereof (Newman and Cragg 2016). Antibiotics that kill or inhibit the growth of pathogenic microorganisms act on a variety of cellular processes, including enzymes involved in the synthesis of the cell wall, proteins and nucleic acids, as well as inhibiting protein degradation and directly damaging DNA. Often, these targets are also present in the antibiotic-producing organisms. As a result, antibiotic producers have evolved various defence mechanisms to avoid damaging themselves through inhibition of these same targets.

Microbially produced antibiotics are typically synthesized by enzymes that are encoded in biosynthetic gene clusters (BGCs). While many genes in these BGCs encode proteins that are associated with biosynthesis and regulation, some have been recognized as providing resistance or immunity to the biosynthesized antibiotics. There are three major BGC-associated resistance strategies that have evolved, considered to represent the origin of antibiotic resistance in natural environments (Davies and Davies 2010): product detoxification; substrate transport and binding; and target duplication or modification. Importantly, this last category allows potential antibiotic-producing BGCs to be prioritized by computational identification of BGC-associated resistance determinants (Tang et al. 2015; Johnston et al. 2016; Alanjary et al. 2017).

Here, we will review the emerging understanding of how antibiotic producers protect themselves during the process of antibiotic biosynthesis, with particular emphasis on target duplication or modification, which can be used experimentally to connect an antibiotic to its mechanism of action. We will discuss how identifying BGC-associated resistance mechanisms allows us to prioritize orphan antibiotic-producing BGCs from DNA sequencing data.

2. BGC-associated self-resistance strategies

NPs produced by microbes often confer a variety of biological activities. For a producer of antimicrobial NPs

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that also contains the target, it must contain gene(s) to provide self-resistance feature(s). There are three main resistance strategies employed by microbes to avoid self-toxicity (Wright 2011): (1) product detoxification, in which the compound is synthesized as, or rapidly converted into, a non-toxic form and only reactivated once it has been released from the cell; (2) binding and removal of the product by high-affinity binding partners and transporters; and (3) target duplication or modification, in which a duplicated or modified target is not susceptible to the product (Figure 1). These resistance genes are generally less favourable for host growth and survival and are thus only expressed concurrently with antibiotic biosynthesis (Andersson and Levin 1999). The most efficient way for bacteria to link these resistance genes, and to ensure efficient co-horizontal gene transfer, is to include the resistance gene within or adjacent to the corresponding antibiotic BGC (D'Costa et al. 2006). However, as the study of NP biosynthetic pathways focuses mainly on the elucidation of the biosynthetic logic and the regulation of the BGCs expression, the resistance genes are often dismissed, as they appear to be unrelated to the surrounding BGC or unnecessary for formation of the NP.

3. Product detoxification for self-resistance

Among these self-resistance strategies, product detoxification is used by many clinically important pathogens to develop resistance to antibiotic treatments. These can be developed *de novo* by recruitment of endogenous enzymes or coopted from environmental strains producing the antibiotics. For example, a chloramphenicol resistance gene from a range of clinical isolates is highly similar to the resistance gene from the producer, *Streptomyces venezuelae*. It is thought these have been mediated by conjugative transfer and transposon-mediated recombination (Jiang et al. 2017). In some antibiotic BGCs, these resistance genes encode enzymes that modify the functional groups of biosynthetic

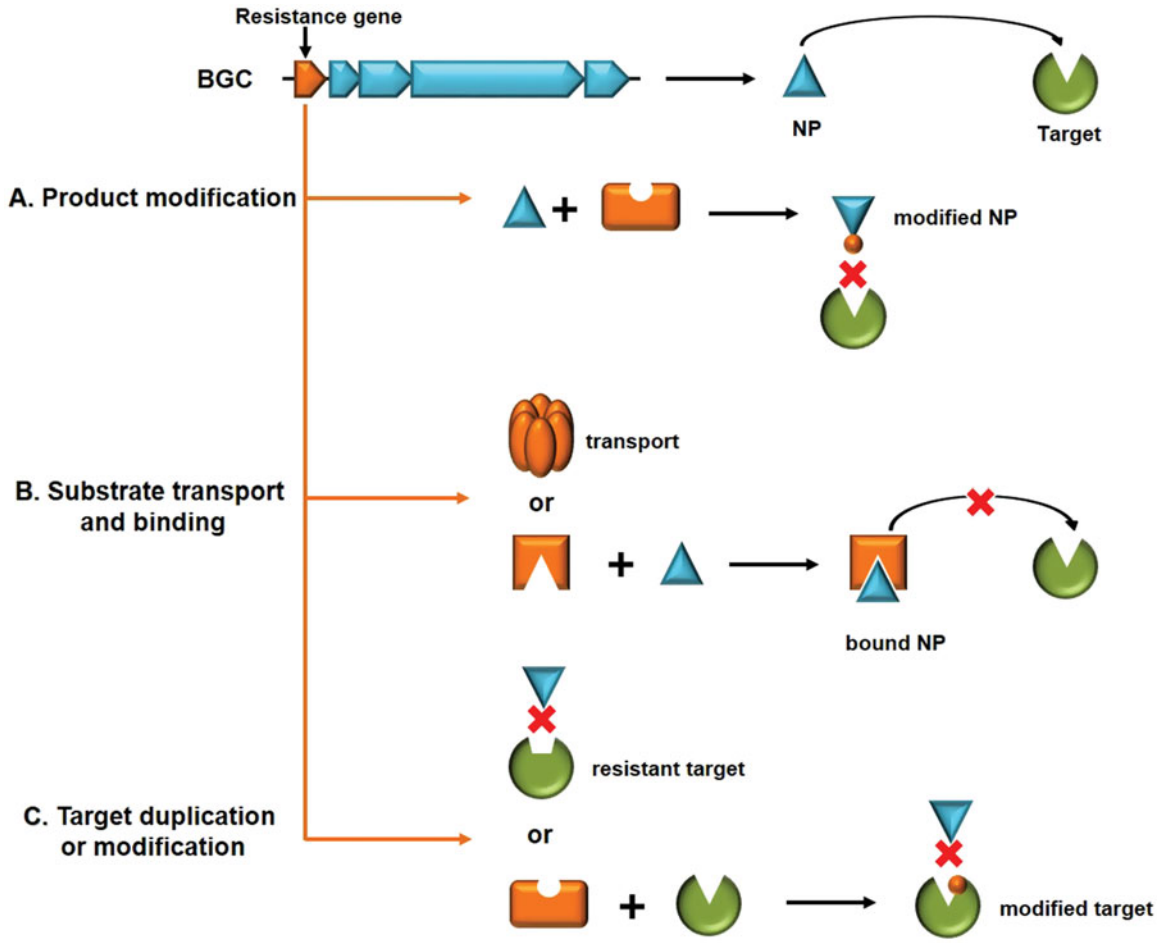

Figure 1. Three BGC associated self-resistance mechanisms. (A) Product modification: the NP is rapidly modified so that it no longer binds to the target; (B) substrate transport and binding: the NP is removed or bound by a transporter or a high affinity binding protein; and (C) target duplication or modification: the BGC encodes a duplicated target resistant to the NP, or an enzyme that modifies the targeting protein so that it is no longer susceptible to the NP.

products by acetylation, phosphorylation, or glycosylation, to prevent binding to the target (Figure 1). For instance, in the kanamycin BGC in *Streptomyces kanamyceticus* is a gene encoding an *N*-acetyltransferase (KanM) which catalyzes the addition of an acetyl group to the C-6′ amine group on the antibiotic, preventing target binding (Kharel et al. 2004).

Capuramycin-type nucleoside antibiotics, including A-500359s and A-503083s, are potent inhibitors of the bacterial translocase I involved in peptidoglycan cell wall biosynthesis. To prevent toxicity in the host strain, an aminoglycoside 3-phosphotransferase is encoded within the capuramycin BGC, which regio-specifically transfers the gamma-phosphate from ATP to the 3′′-hydroxyl of the unsaturated hexuronic acid moiety of capuramycin (Yang et al. 2010). Oleandomycin inhibits protein synthesis by binding the ribosome and inhibiting protein synthesis, much like chloramphenicol and erythromycin (Pestka 1974). In the native producer, *Streptomyces antibioticus*, oleandomycin is glycosylated by *oleD*, a glycosyltransferase encoded in the BGC for oleandomycin biosynthesis (Salas et al. 1994; Quiros et al. 2000).

4. Transport and binding for self-resistance

Perhaps the most ubiquitous method of self-protection is transport by efflux pumps (Figure 1). Genomic analysis revealed that approximately 10% of the transporters in bacteria are involved in multidrug efflux, the largest class in soil or plant-associated microorganism like *Streptomyces coelicolor* (Paulsen 2003). Different efflux protein families have been reported, with the most common being ATP-binding cassette (ABC) superfamily and small multidrug resistance (SMR) superfamily (Li and Nikaido 2009). ABC transporters contain a nucleotide binding domain that is responsible for the binding and hydrolysis of ATP, providing energy for the translocation of the substrate. They also contain a transmembrane domain that forms the translocation pathway for the transported substrates to cross the cytoplasmic membrane (Piddock 2006). In contrast, secondary transporters, like SMR, utilize the proton motive force or ion gradient for drug expulsion (Lubelski et al. 2007).

Nisin is an economically important food preservative agent and antimicrobial lantipeptide produced by lactic acid bacteria, such as *Lactococcus lactis* (Juncioni de Arauz et al. 2009). Characterization of the nisin BGC revealed four genes that encode immunity mechanisms (Cheigh and Pyun 2005). There are three ABC transporter homologues encoded by *nisF*, *nisE*, and *nisG* (Siegers and Entian 1995). The deletion mutants of these genes still maintain the ability to produce nisin, but are more sensitive to its effects. Additionally, NisI is thought to be a protective lipoprotein, showing increased nisin immunity when expressed in sensitive strains, although it is not sufficient for full immunity (Qiao et al. 1995).

Tetracycline prevents protein synthesis by inhibiting aminoacyl-tRNA (aa-tRNA) from binding to the ribosomal acceptor site, rendering it a broad spectrum antibiotic (Chopra and Roberts 2001). Tetracycline resistance is another prime example where two different strategies provide resistance to producing organisms, as well as non-producer pathogens. A multitude of methods for tetracycline resistance has evolved, including efflux pumps (Chopra and Roberts 2001), ribosomal protection proteins (RPPs) (Connell et al. 2003), enzymatic degradation of tetracycline (Speer and Salyers 1989), and rRNA mutations (Ross et al. 1998). Of the 29 identified tetracycline resistance genes, 18 encode for efflux pumps, and 7 encode for RPPs, including rRNA methyl transferases (Chopra and Roberts 2001). The two most studied RPPs, TetO and TetM, can dislodge tetracycline from the ribosome (Connell et al. 2003).

In addition to transport, binding of the toxic molecule can sequester it from the targets, producing another non-specific immune ability of the producer (Figure 1). Bleomycin is a hybrid polyketide-nonribosomal peptide that has been isolated from diverse *Streptomyces* species. It causes nucleotide sequence-specific DNA cleavage and inhibits the growth of both bacterial and mammalian cells. Discovery of the bleomycin BGC from *Streptomyces verticillus* revealed that the protein BlmA is a bleomycin-binding protein, conferring self-resistance by drug sequestration (Kumagai et al. 1999; Shen et al. 2002). A novel method for self-protection was discovered in *Actinomycetes* that produce potent endiynes, which act as DNA-cleaving agents (Biggins et al. 2003). The BGC for calicheamicin, an enediyne antibiotic, was characterized (Ahlert et al. 2002) and the gene *calC*, which encodes a non-heme iron metalloprotein, has been identified as a self-resistance protein (Whitwam et al. 2000). *In vitro* studies showed that calicheamicin abstracts the Gly133 Cα-hydrogen from the CalC metalloprotein, thereby quenching the enediyne and sacrificing the CalC protein (Singh et al. 2006). Two additional proteins, with the same resistance feature, were later identified in the same BGC (Elshahawi et al. 2014). Colibactin is an as-yet-undefined genotoxic small molecule produced by human extra-intestinal pathogenic *Escherichia coli*.

It induces DNA double strand breaks by warhead cross-linking of DNA. Recently, one hypothetical protein produced by the *E. coli* colibactin BGC was characterized as a self-resistance determinant, proposing to be act as a colibactin-sequestering protein (Bossuet-Greif et al. 2016).

5. Target duplication or modification for self-resistance

In order to avoid self-toxicity, producing organisms often duplicate or modify the target of the antibiotics such that they are no longer susceptible. Target duplication or modification uniquely correlates an antibiotic to its mechanism of action (MOA). It is notable that many BGCs contain extra copies of essential genes, which upon closer investigation prove to be resistant to the product (Figure 1). Here we summarize these currently known or proposed self-resistance mechanisms of target duplication or modification, categorizing them based on the MOAs (Table 1).

5.1. Inhibitors of DNA replication and transcription

DNA replication is essential for cell viability and the proteins involved have been identified as potential targets for antibiotics in drug-resistant bacterial pathogens (van Eijk et al. 2017). Although several antimicrobials targeting these proteins have been developed, only gyrase/topoisomerase inhibitors are widely used in the clinic.

5.1.1. DNA polymerase

DNA polymerase is the multi-component protein complex responsible for DNA replication. Griselimycin and its derivatives have long been known to exhibit activity against *Mycobacterium tuberculosis* strains resistant to other antibiotics (Toyohara 1987). The griselimycin BGC, identified in a strain of *Streptomyces*, contained a homologue of DnaN (Broenstrup et al. 2013), part of the DNA polymerase complex that locks the catalytic subunit to the DNA and enhances processivity (Stukenberg et al. 1991) (Table 1). Introduction of this gene into a susceptible strain of *Streptomyces* bestowed resistance to griselimycin, thereby confirming its role in conferring resistance and flagging it as the natural target of the antibiotic (Kling et al. 2015).

5.1.2. DNA gyrase

DNA gyrase is the enzyme responsible for catalyzing the introduction of negative supercoils into DNA, counteracting the positive supercoiling introduced during DNA unwinding by RNA-polymerase (Gubaev and Klostermeier 2014). Originally discovered during the 1950s in a Streptomycete, novobiocin and related aminocoumarins are potent inhibitors of bacterial gyrase (Maxwell 1993), used to treat MRSA infections (Heide 2014). In producer organisms, the housekeeping gene *gyrB* is duplicated, with one of the copies harbouring mutations conferring resistance (Thiara and Cundliffe 1989) (Table 1). Importantly, the resistant copy of *gyrB* was found to be located at the border of the novobiocin BGC (Eustaquio et al. 2005). By using *gyrB* as a probe, the BGC for coumermycin could be identified, validating the hypothesis that gene clusters for other gyrase inhibitors also contain extra copies of this gyrase (Wang et al. 2000). In addition to the *gyrB*, there is also a copy of topoisomerase IV adjacent to this and the clorobiocin gene clusters, providing enhanced resistance (Schmutz et al. 2003).

Fluoroquinolones are synthetic broad-spectrum antibiotic drugs also targeting DNA gyrase and DNA topoisomerase IV. The pathogen *Mycobacterium tuberculosis* employs the protein MfpA, which belongs to the pentapeptide repeat family of proteins (PRPs), to confer resistance (Hegde et al. 2005). This protein mimics the structure of DNA and binds to DNA gyrase, preventing the formation of the fluoroquinolone binding partner, the DNA gyrase-DNA complex. Surprisingly, similar PRPs were later identified as self-resistance elements in the BGCs of two DNA gyrase inhibitors, albicidin (AlbG) (Cociancich et al. 2015), and cytobactamids (CysO) (Baumann et al. 2014) (Table 1). *albG* and *cysO* were furthermore used as queries to search other myxobacteria genomes for orphan BGCs with PRPs acting as self-resistance genes. Eight BGCs were found to have flanking PRPs, and a "silent" Type II PKS BGC was engineered for increased production to reveal the pyxidicyclines, a novel class of topoisomerase I inhibitors, containing an unprecedented nitrogen-containing tetracene quinone scaffold (Panter et al. 2018). This example shows the power of target-directed genome mining in practice to discover new classes of antiinfective agents.

5.1.3. RNA polymerase B

The rifamycins are a group of antibiotics that have been used primarily in the treatment of tuberculosis and other mycobacteria infections (Sepkowitz et al. 1995). This class of compounds was first isolated from

Table 1. Biosynthetic gene clusters associated with target based resistance mechanisms.

Natural product	Target	Organism	Self-resistance mechanism	Self-resistance gene (Accession No)	Ref.
Inhibitors of DNA replication and transcription					
Griselimycin	DNA polymerase sliding clamp	*Streptomyces* sp. DSM 40835	One copy ofDnaN	AKC91855	Kling et al. (2015)
Novobiocin	Gyrase	*Streptomyces niveus* ATCC 23965	One copy of gyrase B	AFI47646	Steffensky et al. (2000)
Clorobiocin	Gyrase	*Streptomyces roseochromogenes* DS 12.976	One copy of gyrase B	AAN65247	Pojer et al. (2002)
Coumermycin	Gyrase	*Streptomyces rishiriensis* DSM 40489	Two copies of gyrase B	AAO47225 AAO47226	Wang et al. (2000)
Albicidin	Gyrase	*Xanthomonas albilineans* GPE PC73	Pentapeptide repeat protein protects gyrase	CBA16025	Vetting et al. (2011); Cociancich et al. (2015)
Cystobactamid	Gyrase	*Cystobacter* sp. Cbv34	Pentapeptide repeat protein protects gyrase	AKP45389	Baumann et al. (2014)
Streptolydigin	RNA Polymerase	*Streptomyces lydicus* NRRL2433	Mutated RNA Polymerase β-subunit	ACL93032	Sanchez-Hidalgo et al. (2010)
Streptovaricin	RNA Polymerase	*Streptomyces spectabilis* NRRL2494	Mutated RNA polymerase β-subunit	AAQ19729	Sanchez-Hidalgo et al. (2010)
Holomycin	RNA polymerase	*Yersinia ruckeri*	One copy of RNA polymerase	hom12	Qin et al. (2013)
Calicheamicin	DNA damaging agent	*Micromonospora echinospora*	Self-sacrificing resistance protein	AAM70338	Ahlert et al. (2002)
Yatakemycin	DNA-alkylating agent	*Streptomyces* sp. TP-A2060	One copy of DNA glycosylase to excise modified base	ADZ13541	Huang et al. (2012); Xu et al. (2012)
Azinomycin	DNA-alkylating agent	*Streptomyces sahachiroi* ATCC33158	One copy of DNA glycosylase to excise modified base	ABY83174	Zhao et al. (2008); Wang et al. (2016)
Colibactin	DNA damaging agent	*Escherichia coli* IHE3034	Self-sacrificing resistance protein		Bossuet-Greif et al. (2016)
Inhibitors of RNA-protein translation and protein synthesis					
Rubradirin	Initiation factor	*Streptomyces achromogenes* var *rubradiris* NRRL 3061	Two copies of translation initiation factor	CAI94679 CAI94684	Sohng et al. (1997)
GE37468	Elongation factor	*Streptomyces* sp. ATCC 55365	One copy of elongation factor	AEM00611	Young and Walsh (2011)
GE2270	Elongation factor	*Planobispora rosea* ATCC 53733	Two copies of elongation factor	AGY49599 AGY49600	Tocchetti et al. (2013)
Erythromycin	23S ribosomal RNA	*Saccharopolyspora erythraea* NRRL23338	One copy of ribosomal RNA methyltransferase	WP_009950391	Dhillon et al. (1989); Vester and Douthwaite (1994)
Pikromycin	23S ribosomal RNA	*Streptomyces venezuelae* ATCC 15439	two copies of ribosomal RNA methyltransferase	AAC69328 AAC69327	Xue et al. (1998); Almutairi et al. (2015)
Thiocillin	Ribosomal protein L11	*Bacillus cereus* ATCC 14579	Two copies of ribosomal protein L11	TclT TclQ	Wieland Brown et al. (2009)
Bengamide	Methionine aminopeptidase	*Myxococcus virescens* DSM 15898	One copy of methionine aminopeptidase	ALK43774	Wenzel et al. (2015)
Fuamgillin	Methionine aminopeptidase	*Aspergillus fumigatus* Af293	Two copies of methionine aminopeptidase	XP_747163 XP_747159	Lin et al. (2013)
Mupirocin	Ile-tRNA synthetase	*Pseudomonas fluorescens* NCIMB 10586	One copy of Ile-tRNA synthetase	AAM12927	El-Sayed et al. (2003)
Cladosporin	Lys-tRNA synthetase	*Cladosporium cladosporioides* UAMH 5063	One copy of Lys-tRNA synthetase	A0A120HYZ1	Cochrane et al. (2016)
Borrelidin	Thr-tRNA synthetase	*Streptomyces parvulus* Tü4055	One copy of Thr-tRNA synthetase	CAE45679	Olano et al. (2004)
Albomycin	Ser-tRNA synthetase	*Streptomyces sp.* ATCC 700974	One copy of Ser-tRNA synthetase	AFJ20776	Zeng et al. (2012)
Agrocin 84	Leu-tRNA synthetase	*Agrobacterium radiobacter* K84	One copy of Leu-tRNA synthetase	ACM31456	Slater et al. (2009)
Indolmycin	Trp-tRNA synthetase	*Streptomyces griseus* subsp. Griseus ATCC 12648	One copy of Trp-tRNA synthetase	AJT38681	Du et al. (2015)
Inhibitors of proteasome					
Salinosporamide A	Proteasome	*Salinospora tropica* CNB440	One copy of proteasome β-subunit	ABP53490	Eustaquio et al. (2009); Kale et al. (2011)
Epoxomicin	Proteasome	*Goodfellowiella coeruleoviolacea* ATCC53904			Schorn et al. (2014)
Eponemycin	Proteasome	*Streptomyces hygroscopicus* ATCC 53709	One copy of proteasome β-subunit	AHB38505	Schorn et al. (2014)
Fellutamide B	Proteasome	*Aspergillus nidulans*	One copy of proteasome β6-subunit	EAA59054	Yeh et al. (2016)

(*continued*)

Table 1. Continued.

Natural product	Target	Organism	Self-resistance mechanism	Self-resistance gene (Accession No)	Ref.
Inhibitors of cell wall biosynthesis					
Vancomycin	Peptidoglycan	*Amycolatopsis orientalis* DSM 40040	Peptidoglycan remodeling	vanH: CCD33128 vanA: CCD33129 vanX: CCD33130	van Wageningen et al. (1998)
Cephamycin	Peptidoglycan	*Streptomyces clavuligerus* ATCC 27064	One copy of β-lactamase	AAF86620	Paradkar et al. (1996)
Cephamycin	Peptidoglycan	*Amycolatopsis (Nocardia) lactamdurans* LC411	Penicillin binding proteins and β-lactamase	CAA78374, CAA78373	Coque et al. (1993)
Inhibitors of fatty acid synthesis					
Andrimid	Acetyl-CoA carboxylase	*Erwinia herbicola* Eh335	One copy of acetyl-CoA carboxyltransferase β-subunit	AAO39114	Liu et al. (2008)
Platencin	FabB/F	*Streptomyces platensis MA7339*	One copy of FabB/F	ACS13710	Smanski et al. (2011); Peterson Ryan et al. (2014)
Platensimycin	FabB/F	*Streptomyces platensis* MA7327	One copy of FabB/F	ADD83010	Smanski et al. (2011); Peterson Ryan et al. (2014)
Thiolactomycin	FabB/F	*Salinispora pacifica* DSM 45543	One copy of FabB/F	ALJ49913	Tang et al. (2015)
Thiotetroamide	FabB/F	*Streptomyces afghaniensis* NRRL5621	Two copies of FabB/F	ALJ49924 ALJ49919	Tang et al. (2015)
Kalimantacin	FabI	*Pseudomonas fluorescens* BCCM_ID9359	One copy of FabI	ADD82948	Mattheus et al. (2010); Mattheus et al. (2010)
Inhibitors of metabolic enzymes					
Phaseolotoxin	Ornithine carbamoyl transferase	*Pseudomonas syringae* pv. actinidiae	One copy of ornithine carbamoyl transferase	BAA19878	Chen et al. (2015)
Mycophenolic acid	IMPDH	*Penicillium brevicompactum*	One copy of inosine 5′-monophosphate dehydrogenase	AJG44383	Regueira et al. (2011)
Lovastatin	HMG-CoA reductase	*Aspergillus terreus*	One copy of HMG-CoA reductase	AAD34556	Hutchinson et al. (2000)
Cyclosporin A	Cyclophilin	*Tolypocladium inflatum*	One copy of cyclophilin	TINF00586	Bushley et al. (2013)
Citreoviridin	F1-ATPase β-subunit	*Aspergillus terreus* var. *aureus*	One copy of F1-ATPase β-subunit	EAU29807	Lin et al. (2016)

the terrestrial actinomycete *Amycolatopsis mediterranei* (Sensi et al. 1959) and later from the marine actinomycete *Salinispora arenicola* (Kim et al. 2006). Rifamycins are a type I polyketide that are synthesized by the assembly of an aromatic starter unit, 3-amino-5-hydroxybenzoic acid through chain extension by propionate and acetate units (Kim et al. 1998). The MOA of rifamycins involves the inhibition of DNA-dependent RNA synthesis in prokaryotes by specifically binding to the β-subunit encoded by the *rpoB* gene (Table 1). The binding is mediated by highly conserved amino acids in the active site of the enzyme, thereby blocking the initiation of transcription.

The rifamycin producers *A. mediterraneii* and *S. arenicola* are known to have mutations in the *rpoB* gene that confer resistance due the decreased affinity of the antibiotic to the enzyme. Most of the mutations occur in regions in the N-terminal half of the *β*-subunit polypeptide. In *E. coli*, these regions are located in amino acids 507–537, 563–572 and near the N-terminus of the *β*-subunit (Floss and Yu 2005). In *A. mediterranei*, the *rpoB* gene is flanking the rifamycin BGC (Floss and Yu 2005), while *rpoB* is not associated with the gene cluster in *S. arenicola* (Freel et al. 2013). Mutated, self-resistant *rpoB* genes are also found in the BGCs for streptolydigin (Horna et al. 2011), streptovaricin (Sanchez-Hidalgo et al. 2010) and holomycin (Qin et al. 2013).

5.1.4. DNA alkylation

DNA alkylating agents display antifungal activity, antibiotic activity, and potent cytotoxicity against different cancer cell lines by causing lesions in the DNA. Yatakemycin (YTM), originally isolated from *Streptomyces* sp. TP- A0356 (Igarashi et al. 2003), contains DNA-binding subunits flanking each side of the central alkylation subunit (in a "sandwiched" arrangement), thereby enhancing DNA alkylation rate and selectivity (Tichenor et al. 2004). The biosynthetic pathway of YTM was identified (Huang et al. 2012), revealing five genes (*ytkR2-R6*) within the BGC that could be responsible for self-defense against YTM. These genes

are considered to be involved in the base excision repair (BER) system (Xu et al. 2012). For example, YtkR2 shows homology with DNA glycosylase proteins from *Bacillus cereus* (AlkD). These enzymes catalyze the first step in the BER pathway by cleaving damaged DNA bases within double-stranded DNA to produce an abasic site (Huang et al. 2012). Further analysis by Xu et al. (2012) provided evidence of the role of YtkR2 as a DNA glycosylase, recognizing and removing the N3-YTM-alkyladenine from the DNA by cleaving the *N*-glycosidic bond to initiate the BER pathway (Xu et al. 2012) (Table 1). Inactivation and expression of an additional copy of *ytkR2* in the native producing *Streptomyces* sp. showed evidence of reduction and increasing yield of YTM, respectively. Although inactivation of the gene does not completely inhibit YTM production, it has been proposed that efflux systems are also involved in the resistance of producers (Xu et al. 2012). Another example is Azinomycin B, a hybrid polyketide/nonribosomal peptide NP which reacts with duplex DNA, introducing interstrand crosslinks (Armstrong et al. 1992), and possesses antitumor activity (Nagaoka et al. 1986). The BGC from *Streptomyces sahachiroi* has been identified (Zhao et al. 2008), and this also contains a DNA glycosylase which provides resistance (Wang et al. 2016) (Table 1).

5.2. Inhibitors of RNA-Protein translation and protein synthesis

The mechanisms of inhibitors of protein synthesis have been a topic of scientific research since at least the 1960s (Vázquez and Kleinzeller 1979). The fundamental nature of protein synthesis lends itself to exploitation by a variety of antibiotics and mechanisms. There are four steps to protein synthesis: initiation, elongation, termination, and recycling, each potential targets for translational regulation and inhibition by antibiotics (Wilson 2014) (Figure 2). The 70S ribosome is made up of the small (30S) subunit and the large (50S) subunit, which are assembled together during initiation. Additionally, the mRNA start codon and initiator tRNA must be accurately positioned to form a functional 70S ribosome. This process is controlled by the translation initiation factors IF1, IF2, and IF3. The elongation cycle is made up of multiple steps, starting with aminoacylated tRNA (aa-tRNA) being delivered by an elongation factor Tu (EF-Tu) to the A site of the ribosome. Additional elongation factors facilitate peptide bond formation, and relocation of the growing peptide chain. Initiation and elongation factors are attractive targets for protein synthesis inhibitors, as well as antibiotics that bind directly to the 30S and 50S subunits (Figure 2).

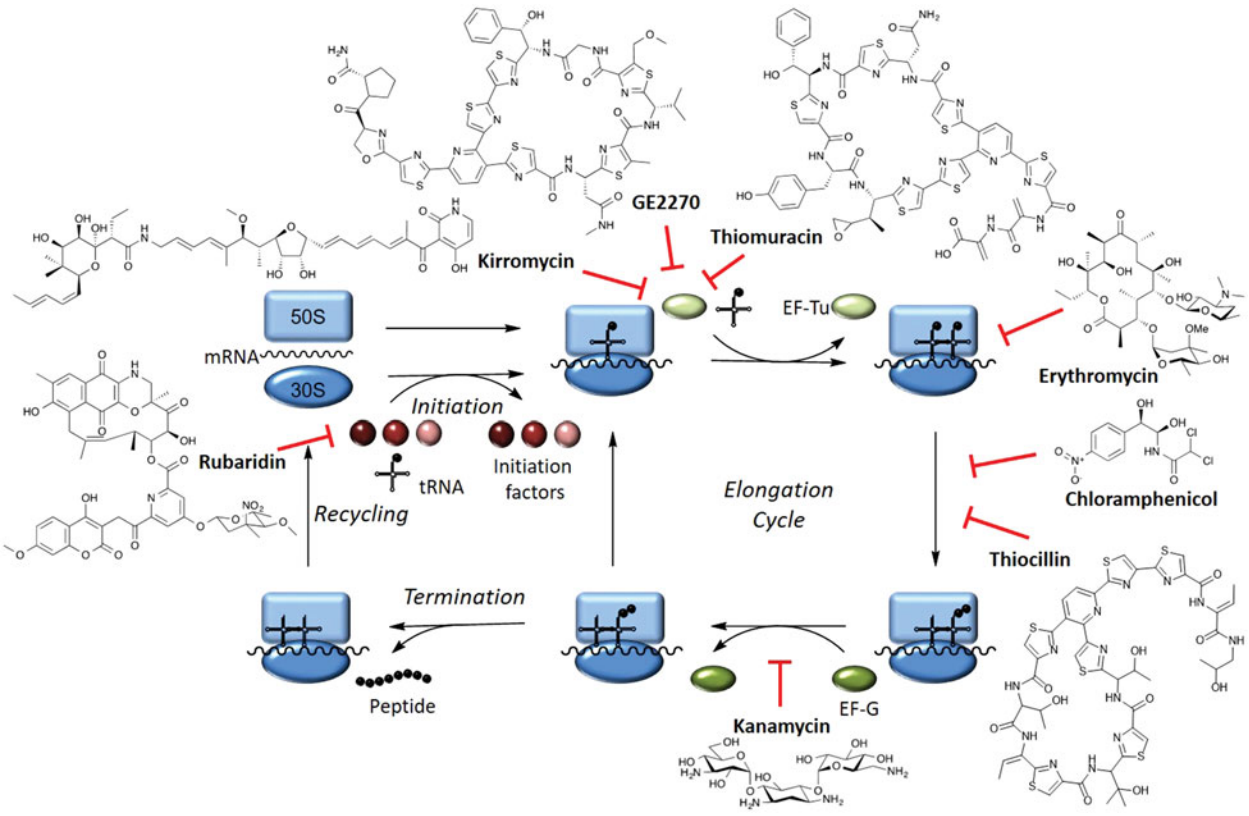

Figure 2. Natural products inhibit the different stages of translation.

5.2.1. Initiation factor

Global regulation of translation occurs mainly through modification of initiation factors (Gebauer and Hentze 2004). Three specialized initiation factors, IF1, IF2, and IF3 control the efficiency and fidelity of the initiation phase (Wilson 2014). Inhibiting these initiation factors from binding is one way to arrest protein synthesis. Rubradirin is an antibiotic, produced by *S. archromogenes* var *rubradiris*, first isolated in 1964 (Bhuyan et al. 1964; Meyer and Mason 1965) and discovered to selectively inhibit initiation factor dependent instigation of protein biosynthesis (Reusser 1983). Rubradirin has a complex molecular structure consisting of four distinct moieties: a quinone, dihydroxydipicolinic acid, a coumarin, and a nitrosugar (Hoeksema et al. 1979). Rubradirin selectively inhibits the binding of tRNA to the 30S subunit if the binding is initiation factor dependent (Reusser 1973) (Figure 2). The BGC for rubradirin was reported in 2008 (Kim et al. 2008). Two translation initiation factors (IF-1) were found within the gene cluster, and are considered to confer resistance, although no biochemical studies have confirmed this yet (Heide 2009) (Table 1).

5.2.2. Elongation factor

Kirromycin is a complex linear polyketide with antibiotic activity against Gram-positive and Gram-negative pathogenic bacteria as well as the malaria parasite *Plasmodium falciparum*. This compound was originally isolated from the actinomycete *Streptomyces collinus* (Wolf et al. 1972). It inhibits bacterial protein biosynthesis by acting on the protein EF-Tu (Figure 2). Binding of kirromycin blocks a conformational shift of EF-Tu when GTP is hydrolyzed to GDP, which prevents the dissociation of EF-Tu from the ribosomal complex, blocking translation (Laiple et al. 2009). The kirromycin producer *S. ramocissimus,* contains three *tuf* genes. Two of these copies are kirromycin-sensitive, however, the third copy (*tuf3)* was shown to be resistant to the antibiotic using an *in vitro* translation system. The kirromycin resistance of EF-Tu3 was explained by replacing the conserved Tyr residue at position 160 with His (Olsthoorn-Tieleman et al. 2007). However, the resistance copy of EF-Tu3 is not located in the kirromycin BGC of *S. ramocissimus*.

Several thiopeptides were identified from *Nonomuraea* strains as inhibitors of EF-Tu (Morris et al. 2009). Elucidation of the thiomuracin biosynthetic pathway revealed a copy of EF-Tu which is encoded by a gene located at the end of the BGC. Additionally, the BGC for the biosynthesis of GE2270, another translation inhibitor, has three ribosomal proteins and two elongation factors, including EF-Tu, downstream (Tocchetti et al. 2013) (Table 1), although these had to be removed before successful heterologous expression could be achieved in *Streptomyces* (Flinspach et al. 2014).

5.2.3. Ribosome

The ribosome is one of the major targets of antibiotics (Poehlsgaard and Douthwaite 2005; Yonath 2005) and a wide range of resistance mechanisms have been utilized by various bacteria (Wilson 2014). To avoid inhibiting their own protein synthesis, many antibiotic producing organisms modify their ribosomes post-translationally by methylation. The classical example of this kind of modification is the Erm methyltransferase contained in the erythromycin BGC. Macrolide antibiotics like erythromycin bind the 23S ribosome through an interaction between residue A-2058 and the 2'OH of the sugar attached to C5 of the lactone ring (Schlunzen et al. 2001). This interaction can be hindered by metabolically shielding the antibiotic as mentioned previously, or by modifying the nucleotide residue to prevent binding. This residue modification is accomplished through dimethylation by specific ribosomal methyltransferases, which represent a large class of enzymes conferring antibiotic resistance, known as "*erm*" genes (Erythromycin Ribosomal Methylation) (Table 1). Other examples include tylosin (*tlrA*, *tlrD*) (Zalacain and Cundliffe 1989, 1991) and carbomycin (*carB*) (Zalacain and Cundliffe 1990). Expression of these genes is tightly regulated as the activity of the erythromycin resistance methyltransferase reduces cell fitness by deregulating translation (Gupta et al. 2013). The ketolide antibiotic pikromycin BGC also contains methyltransferases (Xue et al. 1998), with one (PikR1) being a constitutively expressed monomethylase, providing a low level of background resistance and the second (PikR2), which is an inducibly expressed dimethylase, giving a high level of resistance (Almutairi et al. 2015) (Table 1).

Producer strains of non-macrolide antibiotics that target the ribosome also use nucleotide methylation of the 23S ribosomal subunit to prevent self-toxicity. Although the methylase that confers resistance to the lincosaminide celesticetin targets the same A-2058 residue as the macrolide methylases (Calcutt and Cundliffe 1990), methylation targets are many and varied. Thiopeptides like thiostrepton, siomycin, and nosiheptide have complementary methylases that target the 23S ribosomal subunit at A-1067 (Thompson and Cundliffe 1980; Cundliffe and Thompson 1981;

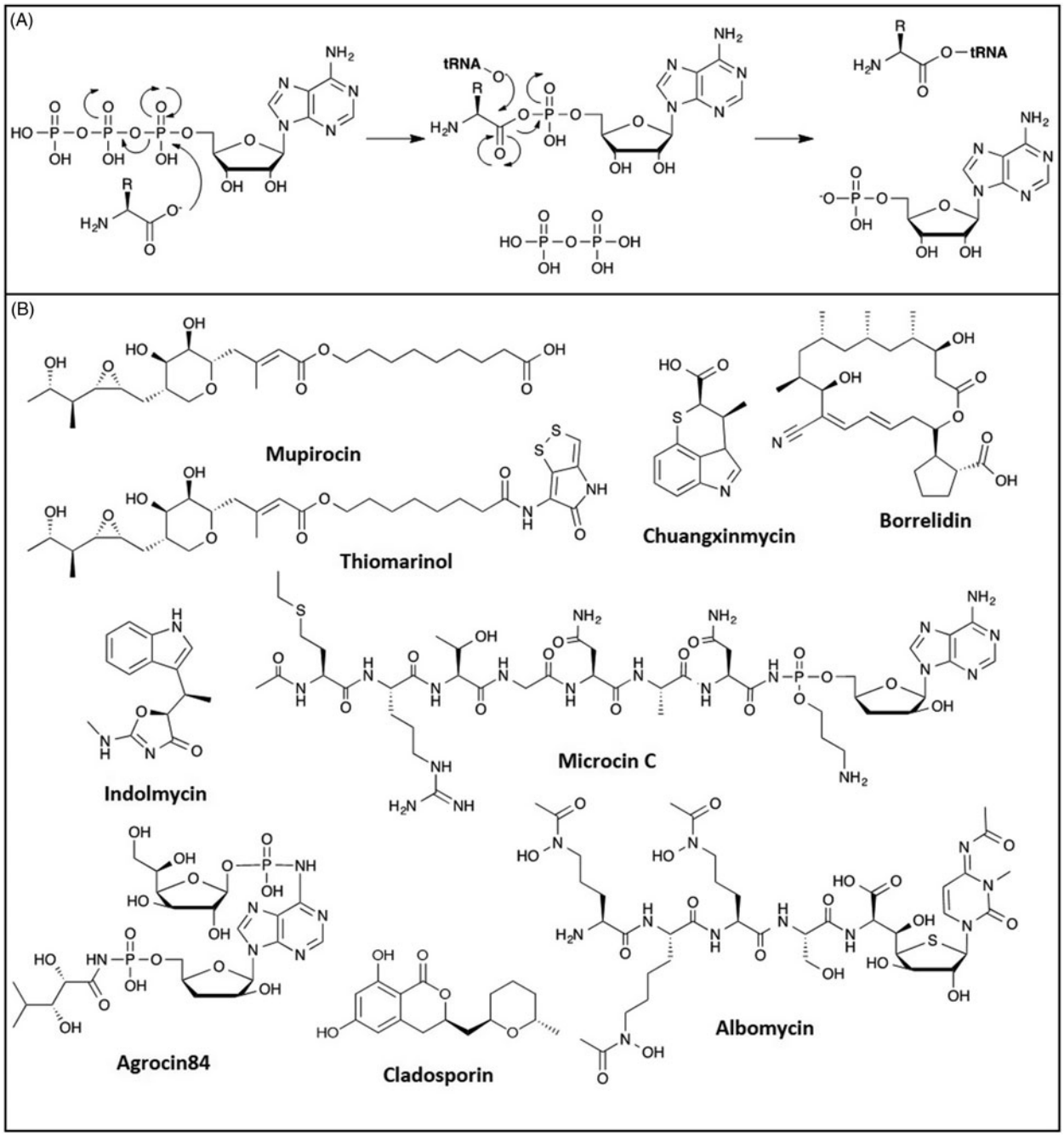

Figure 3. tRNA synthetase reaction and inhibitors. (A) tRNA synthetases catalyze the transfer of amino acids onto their appropriate tRNA via an aminoacyl AMP. (B) Several inhibitors have been identified, showing specificity for particular synthetases. The specificity can be rationalized in some of the structures, such as the indole rings of indolmycin, and the adenyl-AMP warhead of microcin C.

Thompson et al. 1982). The resistance to the orthosomycin antibiotic avilamycin is conferred by two ribosomal methylases (AviRa and AvirRb) (Table 1), which methylate G-2535 and U-2479, respectively (Treede et al. 2003).

Aminoglycosides, pseudodisaccharides, and aminocyclopentitols may also be protected by ribosomal methyl transferases. However, in this case, the 16S subunit of the ribosome is methylated on residues G-1405, A-1408, and G-964 by the protective methyltransferases from the kanamycin (Cundliffe 1992), nebramycin (Beauclerk and Cundliffe 1987), and pactamycin (Ballesta and Cundliffe 1991) gene clusters respectively.

Thiopeptides are highly modified ribosomal peptides (Bagley et al. 2005) which can inhibit the translocation step of protein biosynthesis (Pestka and Brot 1971) by binding the ribosomal L11 protein and the 23S rRNA (Harms et al. 2008). The BGC for the synthesis of

thiocillin was identified in *Bacillus cereus* and encodes two copies of the L11 gene (Wieland Brown et al. 2009) (Table 1), which is proposed to provide resistance to the antibiotic, though this has yet to be experimentally validated. Thus, target modification of the ribosome can be either the modification of the rRNA or by encoding extra copies of ribosomal proteins.

5.2.4. Methionine aminopetidase

Methionine aminopeptidase is required for hydrolytic removal of N-terminal methionine residues from nascent proteins. Bengamides are hybrid PK-NRPs, originally isolated from marine sponges and are of interest as anticancer agents. Analysis of the BGC in a terrestrial *Myxococcus* revealed an additional methionine aminopeptidase (Wenzel et al. 2015) (Table 1). This was shown to provide *E. coli* with resistance to bengamides, and the resistance was localized to a single leucine substitution at position 154 in the protein. Fumagillin is a fungal meroterpenoid, which also targets methionine aminopeptidase and has been used as an antimicrobial agent. The fumagillin BGC was identified in *A. fumigatus* and contains two methionine aminopeptidase-encoding genes, one Type-I and one Type-II, which are proposed to mediate resistance in the producing strain (Lin et al. 2013).

5.2.5. tRNA synthetases

Aminoacyl-tRNA synthetases catalyze the ATP dependent transfer of amino acids to their appropriate tRNA (Figure 3), with unique enzymes for each amino acid (Ibba and Söll 2000). These essential components of the cell machinery can be targeted with potent antibiotics (Agarwal and Nair 2012), and are a target for the treatment of eukaryotic parasites (Pham et al. 2014). These include mupirocin (Hughes and Mellows 1978) (Figure 3), effective against MRSA (Ha et al. 2008), cladosporin, an antimalarial (Hoepfner et al. 2012), and borrelidin (Paetz and Nass 1973) which, in addition to being an angiogenesis inhibitor (Wakabayashi et al. 1997), is together with analogues, a potent antimalarial (Ishiyama et al. 2011; Novoa et al. 2014). The muriprocin producer *Pseudomonas fluorescens* was found to have a resistant Ile-tRNA synthetase (MubM) (Table 1) (Yanagisawa and Kawakami 2003), which was localized in the BGC (El-Sayed et al. 2003). Thiomarinol, a hybrid molecule with a holothin extension on the muriprocin core (Shiozawa et al. 1993), is able to overcome the MubM mediated resistance. Identification of the BGC revealed an alternative Ile-tRNA synthetase (TmlM), which confers resistance to both muriprocin and thiomarinol (Fukuda et al. 2011). The cladosporin gene cluster from the fungus *Cladosporium cladosporioides*, contains a lysyl tRNA-synthetase (Cla4) and two residues have been found to be important for resistance: Gln324 and Thr340, which increase cladosporin sensitivity when mutated (Cochrane et al. 2016).

The BGC for the biosynthesis of borrelidin also has an extra copy of its target, Thr-tRNA synthetase (Olano et al. 2004), though it has yet to be shown to mediate resistance.

Several of the tRNA synthetase inhibitors are substrate mimics, including agrocin84 produced by the biological control agent *Agrobacterium radiobacter* K84 (Reader et al. 2005) and the ribosomally encoded microcin C (Metlitskaya et al. 2006) (Figure 3). The producing strains employ the target modification strategy to protect themselves by synthesizing the antibiotics with an additional peptide, which can enhance the uptake by target organisms. Although the producer of microcin C uses an acetyl transferase to protect itself (Novikova et al. 2010), the BGCs of albomycin and agrocin 84 contain a resistant copy of Ser-tRNA synthetase and Leu-tRNA synthetase (Table 1), respectively (Kim et al. 2006; Zeng et al. 2012).

5.3. Inhibitors of the proteasome

Eukaryotes, archaea, and actinobacteria all possess energy dependent proteasomes, an essential protein-degradation macromolecular complex with archaeal and actinobacterial proteasomes comprising simpler complexes (Bochtler et al. 1999). This large protein complex, called the 26S proteasome, is composed of the core 20S proteasome and the 19S regulatory particle, both made up of multiple subunits (Murata et al. 2009). Proteasome inhibitors (PIs) bind, via a variety of mechanisms, to the hydrolytic β-subunits either irreversibly or reversibly. Bortezomib, a synthetic compound, was the first PI approved by the FDA for treatment of multiple myeloma and mantle cell lymphoma (Figure 4). It was found that repeated exposure to bortezomib caused some cell lines to become resistant through up-regulation and/or mutation of the β_5-subunit in these cell lines (Kale and Moore 2012).

Of the eight structural classes of PIs, five have NPs among them. One example is salinosporamide A of the β-lactone class, produced by the marine actinomycete *Salinispora tropica* (Feling et al. 2003) (Figure 4). It is currently in clinical trials, known as Marizomib, for treatment of multiple myeloma. Insights into PI resistance can be gleaned by studying the biosynthetic pathways of actinomycetes that produce PIs, as they have

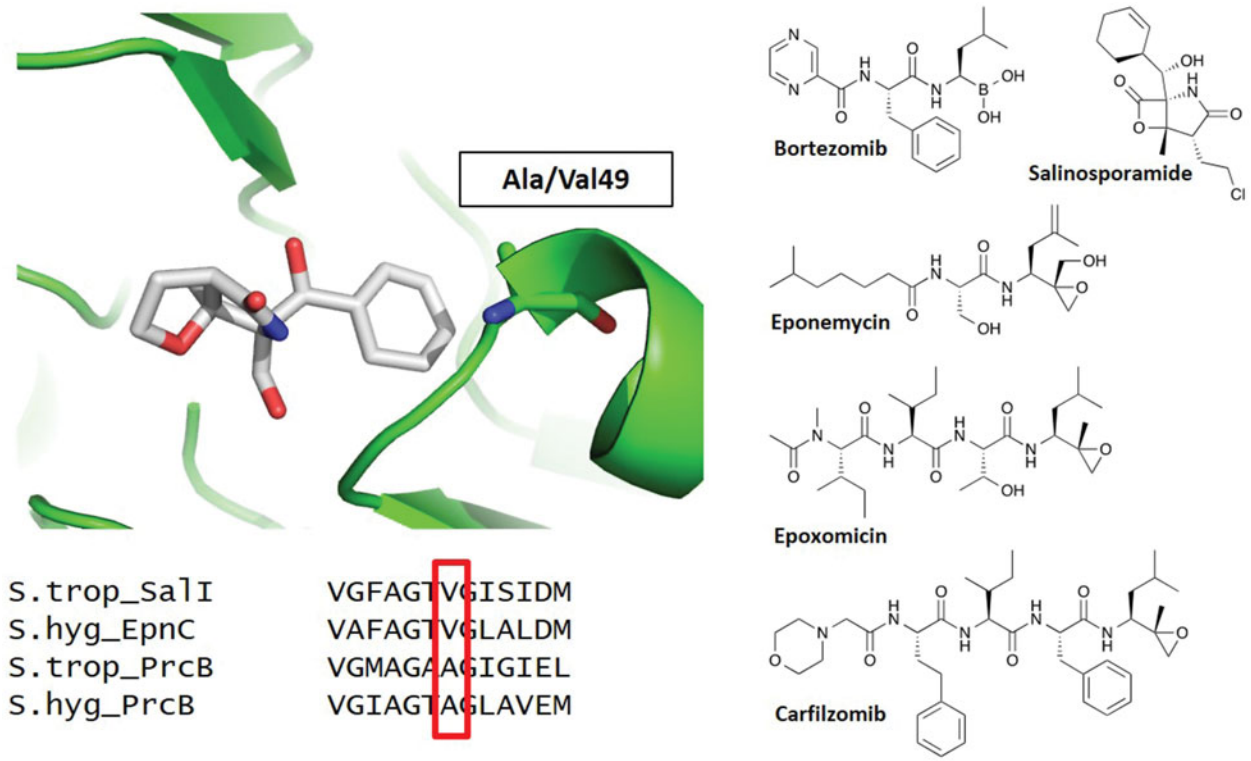

Figure 4. Proteasome inhibitors. Many compounds inhibit the proteasome by binding in the cleft. In the producers of salinasporamide and eponemycin, self-resistance is brought about by an extra copy of the proteasome β-subunits in the BGC which is mutated in this binding cleft from Ala (housekeeping) to Val (resistant).

functioning proteasomes and must have some resistance mechanism to survive. In the salinosporamide A pathway there is an extra gene, *sall*, which encodes for a mutated β-subunit (Kale et al. 2011) (Table 1). This gene is accessory to the normal proteasome machinery in *S. tropica*, and when expressed will complex with the primary α-rings to form a proteasome resistant to salinosporamide A. It was also found to be resistant to bortezomib, suggesting a mutation in the substrate-binding pocket. When comparing the β-subunit S1 binding pocket protein sequence residues of multiple actinomycetes, *Saccharomyces cerevisiae*, and *Homo sapiens*, a mutation at position 49 is apparent (Figure 4). All "non-resistant" sequences contain an alanine at this residue, while Sall has a valine in this position. Site-directed mutagenesis at the 49 position confirmed that a mutation to valine causes a loss in inhibition of the proteasome (Kale et al. 2011).

The most specific and potent class of PIs is the α′β′-epoxyketones, which covalently and irreversibly bind to the proteolytically active subunits of the proteasome. The first two PIs with this unique epoxyketone moiety were epoxomicin (Hanada et al. 1992) and eponemycin (Sugawara et al. 1990) (Figure 4), both naturally produced by actinomycetes. Through whole genome sequencing, it was discovered that both the producers of epoxomicin and eponemycin contain dual proteasome subunits, with mutations in the secondary subunit at position 49 (Schorn et al. 2014). The BGCs for both these compounds were elucidated, and it was found that the eponemycin gene cluster contains the secondary β-subunit, as in salinisporamide A, but the epoxomicin resistant subunit lies outside the BGC (Table 1).

Recently, one orphan BGC (*inp*), which contains a putative proteasome β6 subunit, was revealed by examining uncharacterized *Aspergillus nidulans* BGCs (Bergmann et al. 2010) (Table 1). In order to promote production of the metabolite from *inp*, the promoters of the six genes in the BGC were replaced. This resulted in the production of fellutamide B, a known PI, which could not be obtained from the native stain (Yeh et al. 2016). Thus the previously unknown fellutamide gene cluster was uncovered by targeting the gene cluster containing an interesting target-based resistance mechanism.

5.4. Inhibitors of cell wall biosynthesis

Another target of antibiotic compounds is the cell wall of bacteria, which contains the sugar/amino acid polymer peptidoglycan. Gram-negative bacteria recycle

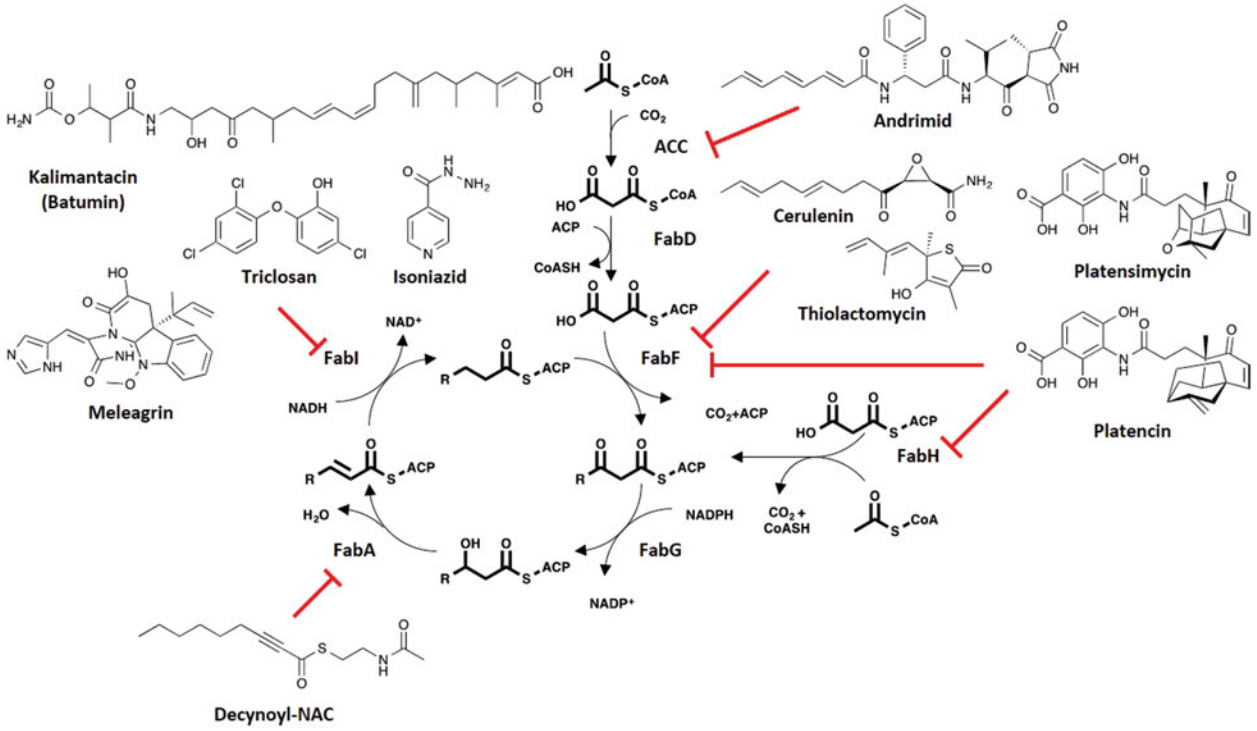

Figure 5. Inhibitors of the enzymes for the biosynthesis of fatty acids. Fatty acids are synthesized via the Claisen condensation of malonate, formed by the ACC catalyzed addition of a CO_2 to an acetyl-CoA, with the growing carbon chain. This is initiated by the FabH catalyzed condensation of acetate and malonate, followed by reduction by FabG, dehydration by FabA, another reduction by FabI before further extension catalyzed by FabF.

30–60% of their cell wall in a single generation (Park and Uehara 2008), making peptidoglycan production a necessary function for living bacteria. One compound interfering with cell wall biosynthesis, vancomycin, was discovered in the 1950s as a metabolite of *Amycolatopsis orientalis* (Pootoolal et al. 2002). Vancomycin inhibits bacterial wall synthesis by binding the carboxyl terminus of D-Ala-D-Ala in growing *N*-acetylmuramylpentapeptide fragments of peptidoglycan (Barna and Williams 1984). Genome sequencing studies have revealed six modes of vancomycin resistance (Pootoolal et al. 2002). In many vancomycin-resistant clinical isolates, expression of a 5 gene cassette (*vanR*, *vanS*, *vanH*, *vanA*, and *vanX*) results in the accumulation of the D-Ala-D-Lac precursor, which serves as an alternative precursor to peptidoglycan and confers resistance to vancomycin (Arthur et al. 1993). In the native producer strain, vancomycin resistance is accomplished by expression of VanA, VanH, and VanX, located at the beginning of the 64-kb BGC (Noda et al. 2004) (Table 1). There are other modifications found in the peptidoglycan of lipid-II targeting antibiotics but these are less well characterized (Stegrnann et al. 2015).

Beta-lactams are another class of antibiotics that inhibit cell wall biosynthesis. Antibiotics like penicillin function by inhibiting the enzymes that cross link peptidoglycan, namely DD-peptidases and DD-transpeptidases, known as penicillin-binding proteins (PBPs) (Sauvage et al. 2008). Resistance to beta-lactams can either be through compound detoxification or through target modification. Enzymes known as beta-lactamases can destroy the structure of the antibiotic by hydrolyzing the beta-lactam ring (Ghuysen 1991). Beta-lactamases are found in the cephamycin BGCs (Table 1), such as in the case of *Streptomyces clavuligerus* (Perez-Llarena et al. 1997). Additionally, this strain produces inhibitors of beta-lactamases, to prevent degradation of their own products by competitors (Ward and Hodgson 1993). Alternatively, mutations in the PBPs can prevent beta-lactam binding and preserve their enzymatic function (Ogawara 2015). PBPs can also be clustered with beta-lactam BGCs, exemplified by *Nocardia lactamdurans*, which clusters the production of cephamycin with a PBP and a beta-lactamase (Coque et al. 1993).

5.5. Inhibitors of fatty acid synthase

Bacterial fatty acid synthase (FASII) is an attractive target for antibiotics, as it is carried out by a series of discrete enzymes rather than the multifunctional enzymes used by animals (Heath and Rock 2004). Different compounds target different steps in this biosynthesis

(Figure 5): enoyl-acyl carrier protein reductase is inhibited by the NP meleagrin (Zheng et al. 2013), and the biomedically important synthetic inhibitors, triclosan (Heath et al. 1999) and isoniazid (Marrakchi et al. 2000); β-ketoacyl-ACP synthase is inhibited by the NPs thiolactomycin (Hayashi et al. 1983) and cerulenin (Dagnolo et al. 1973); and decenoyl-ACP dehydratase/isomerase is inhibited by the synthetic mechanism based inhibitor decynoyl-NAC (Helmkamp et al. 1968).

A high-throughput screening of NPs against the β-ketoacyl-ACP synthase was used to identify the related compounds platensimycin (PTM) (Wang et al. 2006) and platencin (PTN) (Wang et al. 2007) from *Streptomyces platensis* as novel inhibitors of FASII. These compounds, which are derived from terpene and aminobenzoate building blocks (Herath et al. 2007), have been the subject of numerous synthetic efforts to improve their pharmacokinetics (Manallack et al. 2008). The BGC, which was recently identified (Smanski et al. 2011) encodes a FabF homologue (PtmP3), which has since been shown to confer resistance to both molecules (Peterson Ryan et al. 2014) (Table 1). Additionally, the endogenous FabF in the FAS gene cluster was shown to be resistant to both PTM and PTN while the FabH was not resistant to PTN. The authors went on to show that PtmP3 was able to replace both FabF and FabH in the producing organism, indicating this enzyme is able to initiate fatty acid biosynthesis and then extend the acyl chain, activities that have previously only been located in separate enzymes (Peterson Ryan et al. 2014).

A close homologue of the PTN resistance gene was identified within several *Salinispora* and *Streptomyces* genomes. Closer inspection of the genomic region encoding this protein revealed a BGC containing a PKS module and an NRPS module, in stark contrast to the benzoic acid-terpene structure of PTN (Smanski et al. 2011). This BGC was targeted for further study and indeed encodes the biosynthetic machinery for production of the known 3-oxoacyl-ACP-synhtase inhibitor thiolactomycin (Tang et al. 2015) (Table 1). A further homologous gene cluster was identified in *Streptomyces afghaniensis*, containing two copies of the PtnP3 homologue. As predicted, these putative resistance genes provided varying levels of tolerance to these compounds (Tang et al. 2015).

Andrimid is a hybrid polyketide/non-ribosomal peptide with potent antibacterial activity against diverse pathogenic bacteria (Fredenhagen et al. 1987; Needham et al. 2002; Wietz et al. 2011). This antibiotic was originally isolated from the terrestrial Gammaproteobacterium *Enterobacter* sp., a symbiont of the brown planthopper *Nilaparvata lugens* (Fredenhagen et al. 1987). Subsequently, andrimid was isolated from different free-living Gammaproteobacteria from marine environments (Oclarit et al. 1994; Singh et al. 1997; Jin et al. 2006; Wietz et al. 2010). The MOA of andrimid involves blocking the multisubunit acetyl coenzyme A carboxylase (ACC). This enzyme is broadly conserved among bacteria and catalyzes the first step in the fatty acid biosynthesis, which plays an important role in bacterial growth (Freiberg et al. 2004). Andrimid biosynthesis is linked to a 21-gene cluster, including the gene *admT,* with homology to the β-subunit of ACC, which was hypothesized to confer resistance to andrimid for the producing organism (Jin et al. 2006) (Table 1). Walsh and colleagues further investigated this hypothesis by overexpressing *admT* in *E. coli,* which demonstrated that AdmT is an AccD homologue conferring resistance and providing a mechanism of self-protection in andrimid producers (Liu et al. 2008). Mutagenesis and X-ray crystallography of AdmT revealed key mutations that contribute to different levels of andrimid resistance, allowing for the prediction of andrimid resistance among other bacterial strains.

Kalimantacin (batumin) was identified as having antibacterial activity (Kamigiri et al. 1996) and the BGC was identified in *Pseudomonas fluorescens* strain BCCM_ID9359 (Mattheus et al. 2010). This hybrid NRPS-PKS cluster contains a non-biosynthetic enoyl-CoA reductase homologue, BatG (Table 1). This has since been shown to facilitate resistance to the compound, and thus FabI is proposed to be the target of this antibiotic (Mattheus et al. 2010).

Although there is still some doubt as to whether fatty acid synthesis is a clinically useful antibiotic target (Brinster et al. 2009), the widespread use of trichlosan (Russell 2004) and isoniazid (World Health Organization 2010) shows there is great potential in targeting this pathway. PTM also shows antidiabetic effects in a mouse model (Wu et al. 2011), indicating inhibitors of fatty acid biosynthesis may have broader biomedical uses and further effort should be spent on identifying inhibitors of this essential target.

5.6. Inhibitors of metabolic enzymes

5.6.1. Ornithine carbamoyl transferase

Arginine is synthesized by the addition of carbamoyl group from carbamoyl phosphate onto ornithine by Ornithine Carbamoyl Transferase (OCT), forming citrulline, before the addition of nitrogen to form arginine. A tripeptide was isolated from *Pseudomonas phaseolicola*, the causative agent of bean halo blight, and named

phaeseolotoxin (Mitchell 1976). To avoid self-toxicity, this compound is only later cleaved to release the toxic component, which inhibits OCT in plants (Ferguson and Johnston 1980) and bacteria (Staskawicz and Panopoulos 1979). It binds extremely tightly to the enzyme (Langley et al. 2000), although not covalently as originally thought (Templeton et al. 1985). It was found that the OCT in producers were resistant to phaeseolotoxin, as well as the synthetic inhibitor phosphonacetyl-L-ornithine (Mori et al. 1977), but only during production (Staskawicz et al. 1980). This suggested that the organism produces a resistant enzyme during toxin production, which was later identified in the gene cluster for the production of phaeseolotoxin (Aguilera et al. 2007). Thus not only is the producing organism protected from the inhibitor by the shielding amino acids, but also by producing a resistant target.

5.6.2. IMP dehydrogenase

Inosine 5′-monophosphate dehydrogenase (IMPDH) is the first committed and rate-limiting step of guanidine nucleotide biosynthesis. This pathway is present in virtually every living organism, and is heavily upregulated in highly proliferating cells making it an important target for anticancer drugs and antibiotics (Hedstrom 2009). There are currently four approved IMPDH inhibitors: mycophenolic acid (MPA) and mizoribine, which act as immunosuppresents; tiazofurin, an anticancer agent; and the antiviral ribavirin (Morrow et al. 2012). While tiazofurin and ribavirin are synthetic, MPA and mizoribine are both NPs derived from fungi, though only the BGC for MPA has been elucidated (Regueira et al. 2011).

MPA was discovered in 1893, and is credited as the first purified antibiotic from any source (Bentley 2000). Several species of *Penicillium* have been reported to produce MPA (Frisvad et al. 2004), and are simultaneously resistant to its effects, suggesting a specific resistance mechanism encoded in the producing organisms' genomes. A lack of described fungal PKSs producing methylated, non-reduced products, as would be expected for MPA, forced the authors to explore other strategies for finding the MPA gene cluster. Instead, they used IMPDH as a search strategy, hypothesizing a homologue of IMPDH would be included in the gene cluster. This approach leads the researchers to discover the IMPDH homologue *mpaF* in the full MPA biosynthetic pathway of *Penicillium brevicompactum* (Regueira et al. 2011) (Table 1).

When heterologously expressed in a susceptible fungus, *Aspergillus nidulans*, MpaF conferred resistance to MPA (Hansen et al. 2011). Furthermore, *mpa*F was used as a probe to search six MPA producing or non-producing strains of *Penicillium*, all of which were found to contain dual homologues of IMPDH. Phylogenetic comparison of these IMPDH genes revealed distinct clades for the resistant IMPDH genes and the primary IMPDH genes. Additionally, a mutation of the residue at position 415 in the resistant version, from tyrosine to phenylalanine, is observed, although it is not known if this mutation is responsible for conferring resistance, as position 415 is not in close proximity to the MPA binding site. The identification and localization of the MPA BGC by searching for a resistance gene residing in the cluster is a prime example of the power of using such biological markers for discovery of biosynthetic genes based on target modification.

5.6.3. HMG-CoA reductase

Lovastatin is a fungal-derived polyketide inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor and, in addition to being used as a chlosterol lowering statin, is also an antifungal (Chamilos et al. 2006). To prevent toxicity in the producing strain, the lovastatin BGC of *Aspergillus terreus* was found to encode an additional HMG-CoA reductase (*lvrA*) (Table 1), which confers resistance to lovastatin when expressed in sensitive strains (Hutchinson et al. 2000). This additional copy of an HMG-CoA reductase is found in related statin BGCs and can be readily identified using bioinformatics.

5.6.4. Cyclophilin

Cyclosporin A is an immunosuppressant which inhibits cyclophilins, enzymes which catalyze the isomerization of the proline bonds during protein maturation. The BGC for the biosynthesis of cyclosporine was identified in the producing fungus *Tolypocladium inflatum* and was found to contain an additional cyclophilin, which was upregulated during cyclosporine production (Bushley et al. 2013). This indicates that a resistant copy of the enzyme is produced alongside the inhibitor, though this has not been formally characterized (Table 1).

5.6.5. F1-ATPase

Citreoviridin is a polyketide inhibitor of F1-ATPase β-subunit which is under investigation as an anticancer agent (Gause et al. 1981). The BGC was identified in *Aspergillus terreus* by identifying an extra copy of the F1-ATPase β-subunit next to a suitable PKS gene cluster, followed by heterologous expression (Lin T-S et al. 2016). *Metarhizium anisopliae*, a known producer of the

structurally similar toxin aurovertin, also harbours a cluster containing a β-subunit of ATP synthase, and genes in the cluster have homology to the citreoviridin locus (Azumi et al. 2008) (Table 1).

5.6.6. Squalene synthase

Squalestatin, an inhibitor of squalene synthase, shows broad-spectrum antifungal properties (Dawson et al. 1992). Identification of the BGC for squalestatin S1 in three different producing strains of fungi, revealed the presence of a squalene synthase, R6, which is presumed to be a resistance protein (Bonsch et al. 2016).

6. Target-directed genome mining

The ability to connect natural antibiotics to gene clusters and vice versa, along with ever-increasing knowledge of biosynthetic logic, has spawned a new field of NP genome mining for the rational discovery of new chemical entities. Recent advances in genome sequencing technologies have revealed that only a small fraction of the NP biosynthetic potential of most microbes has been uncovered using traditional approaches. This not only provides unprecedented opportunities to further explore new compounds but also suggests that current methods have grossly underrepresented the chemical breadth of secondary metabolism in microorganisms. One of the most daunting tasks in this field is how to prioritize orphan BGCs that may produce molecules with favourable bioactivities, especially when selecting amongst the tens of thousands in public databases.

Recently, it has been shown that searching for putative resistance genes within BGCs could provide insight to the molecular targets of BGC chemical products prior to their structure elucidation and mechanism of action studies, in a process called target-directed genome mining (TDGM) (Tang et al. 2015). Tang et al. initially identified groups of related protein-coding genes that are shared amongst a species as core housekeeping genes and additional copies of these genes were then identified in the genomes, with those in BGCs presumed as potential target based resistance genes. This strategy not only identifies known resistance genes, but can potentially uncover novel resistance genes for targets against which no NPs have been characterized. 912 duplicated housekeeping genes were identified within BGCs in the genomes of 86 strains of *Salinispora*, across a wide range of functional categories of proteins. This successfully identified the 20S proteasome β-subunit gene within the saliniporamide BGC, which confers resistance to the product. One of these duplicated housekeeping genes was an extra copy of the fatty acid biosynthesis enzyme 3-oxoacyl-ACP-synthase, found within an unusual hybrid-NRPS-PKS BGC in the genome of *Salinispora pacifica* CNS-863. Cloning and heterologous expression of this BGC showed that the production of a series of unusual thiotetronic acid NPs, including the FASII inhibitor thiolactomycin (Tang et al. 2015), which was first described over 30 years ago yet never connected to its BGC. The identification of the large number of target-based resistance genes within BGCs is greatly facilitated by the extensive classification in the genus *Salinispora*. However, the same strategy could be applied to any sufficiently well-sequenced group of related species.

Alternatively, a target agnostic strategy could be done by taking a bottom-up approach, in which individual BGCs are first identified, and then each gene interrogated for its likely function, identifying known resistance genes, such as those described in this review. These can then be phylogenetically compared to housekeeping genes from the host organism and close relatives, as they often do not clade with their parent organism, an indication that these have been acquired under a significant selection pressure (Freel et al. 2013).

A recent bioinformatic tool, the Antibiotic Resistant Target Seeker (ARTS) (Alanjary et al. 2017) has automated TDGM in a user-friendly web interface (http://arts.ziemertlab.com). ARTS identifies known resistance factors and duplicated housekeeping genes within a genome, determines their proximity to BGCs, and builds phylogenetic trees to highlight incongruent phylogeny suggesting horizontal transfer. Importantly, ARTS not only highlights known resistance targets, but expands this search to putative targets, without any known antibiotics acting against them, that meet the resistant target criteria. Computational automation of TDGM now allows for high-throughput BGC prioritization and possible new target identification.

It should be noted that for biosynthetic enzymes it is difficult to determine if the copy in a BGC is a resistance mechanism, as opposed to playing a direct role in the synthesis of the small molecule. For example, Ser-tRNA synthetase acts as a resistance mechanism in the albomycin BGC (Zeng et al. 2012), but in the biosynthesis of valanimycin a protein with homology to Ser-tRNA synthetase is used to transfer serine from Ser-tRNA onto the growing antibiotic (Garg et al. 2008). Identified BGCs should be compared to homologous BGCs and to gene orthologue neighbours of the putative resistance gene. If highly homologous BGCs can be found which lack the putative target gene, this indicates it is not necessary for biosynthesis and other resistance

mechanisms may be used, or a resistant copy of the housekeeping gene is found outside the cluster, as is the case for the eponemycin gene cluster (Schorn et al. 2014), Sometimes, similar resistance genes could be located in radically different BGCs, indicating the products may target the same proteins. For example, the self-resistance proteins produced by thiotetronic acid antibiotic BGCs showed high similarity to the resistance determinants PtmP3 and PtnP3 from the PTM and PTN BGCs. During the discovery of the thiolactomycin BGC, we recognized that many homologues (>60% identity) of the FASII self-resistance protein could be identified in various locations in different *Streptomyces* genomes, many related to BGCs (Tang et al. 2015). This indicates that more BGCs for production of FASII inhibitors could be identified in the public database.

7. Conclusions

Antibiotics are of paramount importance as weapons against a variety of pathogenic bacteria. However, the use of antibiotics selects for resistant organisms and marginalizes many clinically important antibiotics. Resistance can naturally exist in native antibiotic producers and emerge within pathogenic bacteria by mutation or by horizontal gene transfer from other organisms. Resistance mechanisms in pathogens have been extensively discussed in various review articles (Wright 2011) (Davies and Davies 2010). By comparison, BGC-associated resistance genes from environmental strains appear to be less intensively described in the literature. Indeed, such environmental strains are the origin of antibiotic resistance in natural environments. How does nature lead to the evolution and dissemination of antibiotic resistance genes from producing organisms? What are the physiological and biological roles of antibiotics in the producing microbes and microbe–microbe or microbe–host interaction? To address these questions largely depends on a detailed examination of these genes.

Modern genomics reveals a huge diversity of NP BGCs, much broader than the glimpse shown by traditional growth and purification. Utilizing modern techniques, it is possible to access these clusters, either by switching them on in their hosts or by transferring them to other expression hosts. By selecting BGCs that have extra copies of self-resistance housekeeping genes, bioactive compounds can rapidly be discovered with a strong hypotheses on their MOA. This review has covered known targets and delineated the discovery process for several new compounds using TDGM. However, there are potential BGCs which contain targets for which there are no known inhibitors. These represent the most valuable BGCs for uncovering new antibiotics against new targets, with minimal clinical resistance.

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Disclosure statement

The authors report no conflicts of interest.

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