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Self-Resistance of Natural Product Producers: Past, Present, and Future Focusing on Self-Resistant Protein Variants

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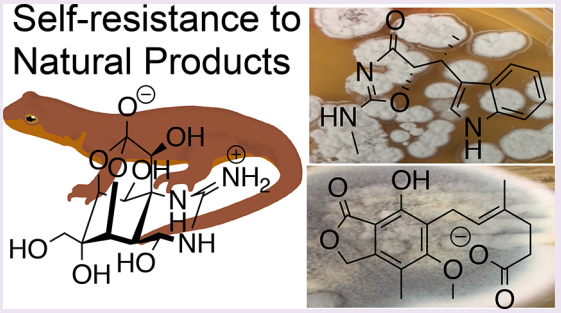
Supporting Information

ABSTRACT: Nature is a prolific producers of bioactive natural products with an array of biological activities and impact on human and animal health. But with great power comes great responsibility, and the organisms that produce a bioactive compound must be resistant to its biological effects to survive during production/accumulation. Microorganisms, particularly bacteria, have developed different strategies to prevent self-toxicity. Here, we review a few of the major mechanisms including the mechanism of resistance with a focus on self-resistant protein variants, target proteins that contain amino acid substitutions to reduce the binding of the bioactive natural product, and therefore its inhibitory effects are highlighted in depth. We also try to identify some future avenues of research and challenges that need to be addressed.



Natural products (secondary metabolites) have had a great impact on human civilization throughout history and have been derived from plants, animals, and microorganisms. These small molecules (typically less than 2000 molecular mass) have served and continue to serve as drugs and drug leads that are important in human health and agricultural practices. Currently, greater than 50% of clinically used and approved drugs are either from natural products origin or inspired from a natural product pharmacophore.[1−3] In agriculture, natural products play an important role in pest control (*e.g.*, spinosyn).[4,5] Given their importance in human civilization much effort has been put toward understanding their biological activities and biosynthesis.

Traditionally natural products have been studied using bioactivity guided fractionation, in which plant or animal material, microorganism growth media, or a microorganism itself is chemically extracted, and the extract is screened in a biological assay. The extract is then fractionated until pure compounds with the desired biological activity are isolated. These small molecules are then structurally characterized using 1D and 2D NMR spectroscopy, mass spectrometry, and IR spectroscopy among other methods. Biological assays began with simple cell death assays, including the disk diffusion assay, evolved to *in vitro* assays screening single protein targets during the revolution in recombinant DNA technology, and recently have returned to cell death assays complemented by high content imaging.[6] New techniques for overcoming the problem of discovery of known compounds (dereplication) and discovering new analogs have been pioneered including mass spectrometric networking.[7]

In recent years, the drastic decrease in cost of genome sequencing, genome assembly, and annotation has led to a renaissance in natural products research by demonstrating that microorganisms produce only a fraction of the natural products that they encode biosynthetic gene clusters (BGCs) for under laboratory culture conditions.[8] These orphan clusters have been activated by using techniques such as promoter exchange,[9] and the one strain, many compounds (OSMAC) approach.[10] For a more comprehensive review, see the recent reviews by Rutledge and Challis[11] and Zhao and co-workers[9] and references therein.

But how to deal with the large amount of genomic data that is being generated and how does one prioritize clusters for investigation has now become a major focus. Recently, algorithms that cluster BGCs,[12−14] algorithms that compare the predicted products to known molecules,[15] and algorithms clustering molecules and the mechanism of action have been developed to aid in prioritiziation.[16] In parallel, researchers have used self-resistance to enrich microbial producers of a particular class of compounds (*e.g.*, glycopeptides) in an effort to increase the probability of discovering new compounds in the same class.[17] Recently, a web-based tool named the Antibiotic Resistant Target Seeker (ARTS) has been released to help prioritize microbial strains by identifying those with the potential to produce a natural product with a desired mechanism of action (*vide infra*).[18]

Previous Reviews. Self-resistance is a necessary survival adaptation to the production of bioactive natural products. If the producing organism contains the targeted pathway or cellular components, then it is required that it evolves a way to prevent self-toxicity. Self-resistance has been studied in the context of antibiotic producing microorganisms since natural products have been investigated, in part because these resistance mechanisms are thought to be a reservoir of resistance determinants observed in clinical settings.[19] The reader is directed the to Comprehensive Antibiotic Resistance Database (CARD) where an up-to-date listing of clinically relevant antibiotic resistant mechanisms

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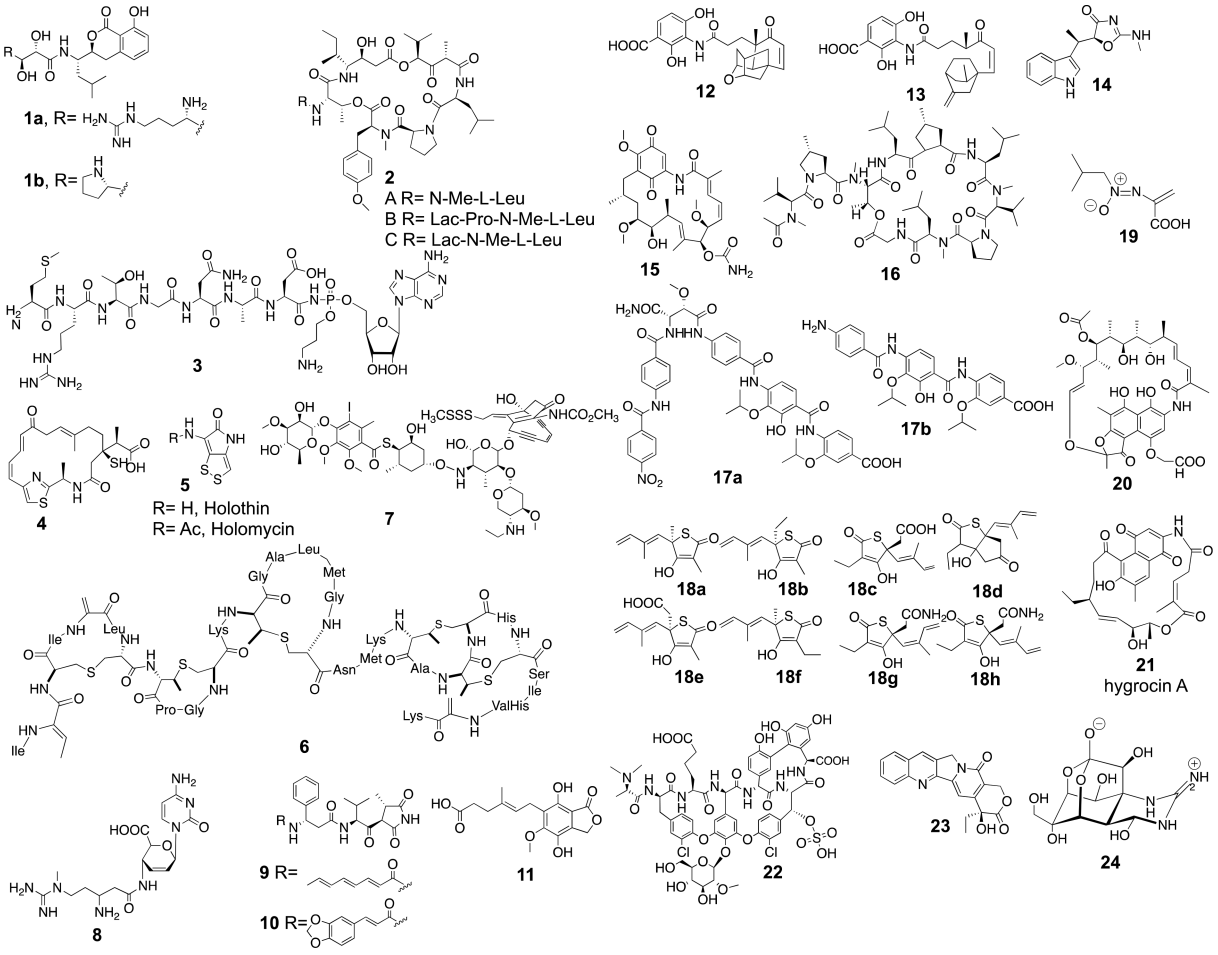
is maintained.[20] In addition, many of these self-resistance genes have been adapted for the benefit of molecular biological researchers, allowing them to select recombinant DNA after transformation. The reader is referred to previous reviews that provide a background of previously studied systems authored by Cundliffe and Demain,[21,22] Hopwood,[23] and Wright.[19,24] For a review of the regulation of self-resistance, the reader is directed to a more in-depth review authored by Nodwell and co-workers.[25]

Here, we describe some examples of self-resistance to natural products including bacterial, fungal, plant and vertebrate examples; how they were utilized in natural products research; and the potential for self-resistance to enhance research in the field of natural products and drug discovery.



INTRODUCTION—TYPES OF SELF-RESISTANCE

Organisms that produce toxic natural products must be resistant to the action of these toxic agents in order to survive and gain an advantage when these compounds are produced/released. There are six major strategies that organisms utilize alone or in combination (Figure 1).

Efflux Pumps. One of the most common strategies is the use of efflux pumps (e.g., ATP-binding cassette (ABC) transporters). These transmembrane proteins are found in many natural product BGCs and are also involved in antibiotic resistance in the clinical setting as recently reviewed by Mousa and Brunner.[26]

Chemical Modification. Another strategy that is widespread is chemical modification (also known as metabolite inactivation) of the natural product, either through addition of a chemical entity (*e.g.*, phosphate, acetate) or degradation. A large number of the genes/proteins responsible for chemical modification of natural products have been co-opted by scientists to help in the selection of plasmids and genetic transformants (*e.g.*, chloramphenicol acetyl transferase, β-lactamase, aminoglycoside phosphotransferase).

Prodrugs. The production of prodrugs, which are converted to the active compound during excretion or uptake by a competing organism is another avenue for self-protection. Lantibiotics,[27] colibactin (produced by *Escherichia coli*),[28] xenocoumacin (**1**a and b; produced by *Xenorhabdus nematophila*),[29] and didemnins A, B, and C (**2**; produced by *Tistrella* sp.)[30] are examples of natural products that are produced in a prodrug form, and the active form is released upon excretion from the producing cell. In contrast, prodrugs that are activated after uptake by cells encompass Trojan horse antibiotics such as microcin C7 (**3**)[31] but also include compounds activated by radical oxygen species such as leinamycin (**4**)[32] and compounds activated by disulfide reduction such as the dithiolopyrrolones (**5**).[33,34]

Compound Sequestration. Sequestering an active molecule from binding to its target has been observed as a resistance mechanism for natural products. The sequestration of the natural product is typically accomplished by binding to a protein encoded in the BGC. The biding can be reversible as in the case of nisin (**6**) and the self-protection protein NisI, wherein binding of nisin prevents it from binding to lipid II.[35,36] Alternatively, the binding can result in the degradation of the binding protein as exemplified by interaction between CalC and calicheamycin γ_1^I (**7**).[37] In this example, calicheamycin γ_1^I is bound by CalC,

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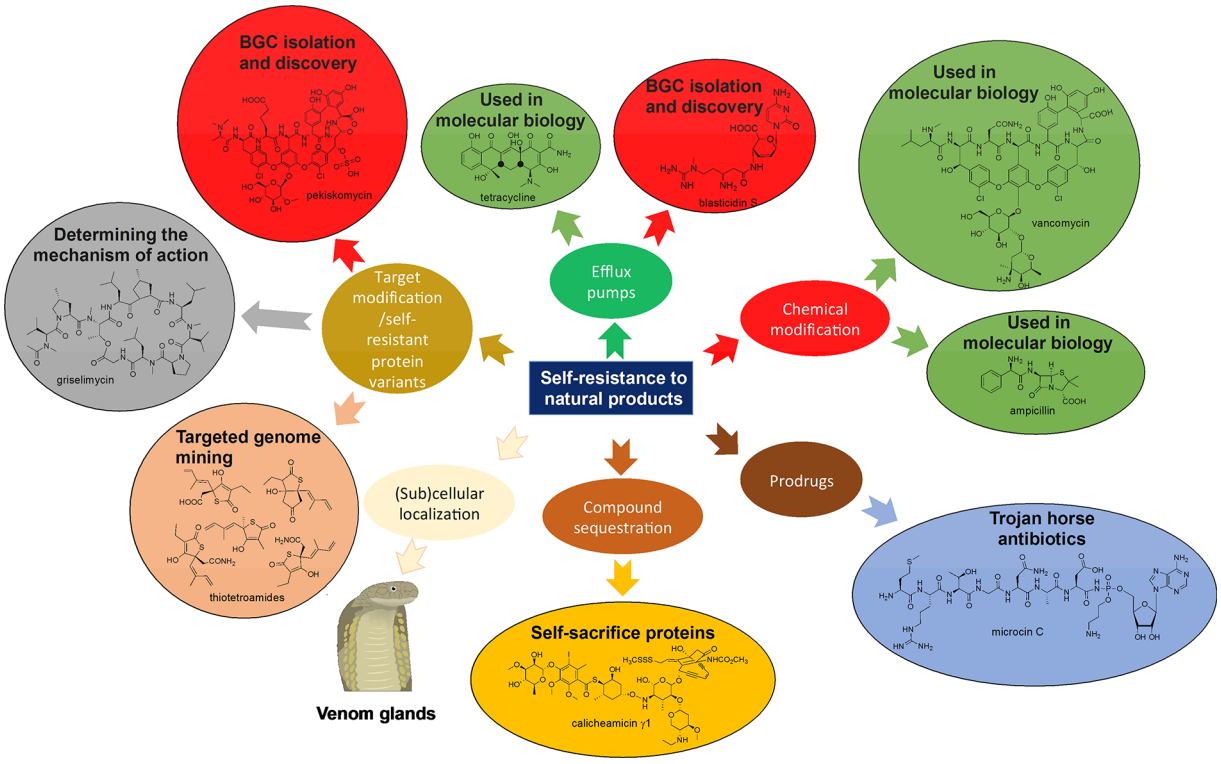

Figure 1. Overview of self-resistant strategies utilized by organisms. The six major strategies utilized by organisms noted in this review are listed in the inner ring, while examples of how these strategies have been used in natural product and molecular biology research and selected examples are shown in the outer ring.

and upon activation of the ene-diyne warhead, which forms the reactive 1,4-benzyl diradical species, a hydrogen atom is removed from Gly_{113} of CalC. This hydrogen abstraction, plus an additional hydrogen abstraction from a water molecule, quenches the 1,4-benzyl diradical species, preventing abstraction of a hydrogen atom from DNA which results in DNA cleavage. Bleomycin, mitomycin, and the nine-membered ene-diyne natural product family, all potent DNA damaging agents, have a common self-resistance mechanism analogous to that seen in the above-described example for CalC/calicheamicin as reviewed previously by Shen and co-workers.[38] Both the bleomycin producer, *Streptomyces verticillus*, and the mitomycin producer, *Str. lavendulae*, encoded proteins which bind the natural products, thereby sequestering them from DNA. Nine-membered ene-diynes BGCs also encode a binding protein (*e.g.*, NcsA, neocarzinostatin A), which seems to function as both a sequestration mechanism and one involved in stabilizing the reactive nine-membered ene-diyne core.

(Sub)cellular Localization. An alternate strategy is to sequester the active natural product away from the biological target. This is a strategy more frequently used by eukaryotic organisms. A macrostructural example is the venom glands of snakes, which sequester the neurotoxic components of venom away from the neural system of the snake. On a cellular level, fungi are known to concentrate compounds in subcellular compartments or vesicles. As reviewed recently by Kistler and Broz there are unresolved questions on whether these vesicles are to solely quarantine toxic intermediates or provide environments where proteins and precursors could be gathered to facilitate biosynthesis.[39] A similar situation is found in plants, with the best-studied example being the vinca alkaloid producer *Catharanthus roseus*.[40,41]

Target Modification/Self-Resistant Protein Variant. Alternatively, the target (protein or RNA) that binds the natural product can be altered to prevent binding and thereby confer resistance. A well-known example is the modification of the ribosome by methylation, a strategy that has resulted in resistance to aminoglycosides and was recently reviewed by Vester and Long,[42] Morić *et al.*,[43] and Wilson.[44] An alternate strategy that been recognized in a small but growing number of BGCs is encoding a version of the protein target with amino acid substitutions in the active site which impair binding of the natural product but do not abolish catalytic activity, resulting in a self-resistant protein variant. As a general feature, these self-resistant protein variants are encoded in the BGC as a second copy. The housekeeping copy is encoded in a different locus of the genome and is usually sensitive to inhibition but typically has higher catalytic turnover compared to the self-resistant protein variant. This allows the organism to use the catalytically optimized protein during growth and only express the self-resistant protein variant when needed in coordination with production of the inhibiting natural product. It is this final class of resistance proteins that is the focus of this review.

USE OF SELF-RESISTANCE IN NATURAL PRODUCT BGC ISOLATION AND DISCOVERY

Blasticidin S. Blasticidin S (**8**) is a peptidyl nucleoside antibiotic that was first isolated from *Str. griseochromogenes*.[45] Blasticidin S inhibits protein synthesis by blocking the termination step of protein translation.[46] Cone *et al.* used this selective pressure to identify genomic DNA fragments that allowed a blasticidin-sensitive strain of *Str. lividans* to grow in the presence of up to 600 μg/mL blasticidin S. The screening revealed two DNA fragments (2.6 and 4.8 kb) that encoded

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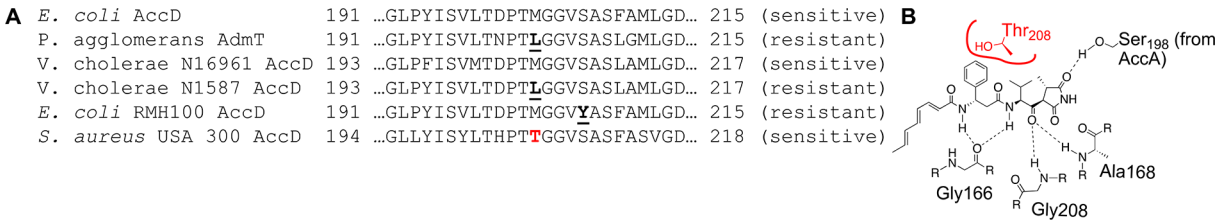

Figure 2. Interaction between andrimid and AccD. (a) Partial amino acid sequence alignment of AccD/AdmT from *Escherichia coli*, *Vibrio cholera*, *Pantoea agglomerans*, and *Staphylococcus aureus*. The bolded and underlined amino acids are those found in andrimid-resistant variants of AccD/AdmT and were shown to be responsible for conferring resistance to andrimid. *E. coli* RMH100 was isolated as a spontaneous resistant mutant to andrimid analog **10**. The underlined and bolded residue (Tyr205) was shown to be responsible for reduced binding of andrimid to the AccD subunit. The listing of resistant or sensitive in parentheses indicates whether the organism is resistant or sensitive to andrimid. (b) Schematic diagram of the predicted interactions of andrimid with *Sa*AccD (PDB 5KDR). The substitution of Thr208 (red) by leucine creates a steric clash between the side chain of leucine and the phenyl group of andrimid. Hydrogen bonds are indicated by dashed lines.

resistance genes.[47] These DNA fragments were then used to screen a cosmid library to identify the full-length cluster. Interestingly, only cosmids that hybridized to the 4.8 kb fragment were capable of producing blasticidin S and precursors (e.g., cytosylglucuronic acid) upon introduction to Str. *lividans* TK24.[47] Subsequent research led to the reporting of the blasticidin S BGC and the identification of *blsJ* as the gene involved in the resistance to blasticidin S described above. BlsJ was predicted to have 11 membrane-spanning domains and was predicted to be part of ATP-binding cassette (ABC) transport system involved in the efflux of blasticidin S.[48] With the identification of the BGC and boundaries, further biosynthetic studies characterizing key steps and biosynthetic enzymes were then undertaken.[45,48−52]

Andrimid (9). An alternate but related approach was taken by Clardy and co-workers during the identification of the BGC involved in andrimid biosynthesis. Andrimid is a hybrid nonribosomal peptide-polyketide that exerts its antibiotic activity through the inhibition of fatty acid biosynthesis by blocking the carboxyltransfer reaction carried out by acetyl CoA carboxylase (ACC).[53] As *Escherichia coli* is sensitive to andrimid, a cosmid library was constructed from the genomic DNA of *Pantoea agglomerans* strain Eh335 (native andrimid producer). This cosmid library was introduced into *E. coli*, and the colonies were selected on both chloramphenicol (selection of the cosmid vector backbone) and andrimid. This led to the identification of clones that contained the full biosynthetic gene cluster. This screening was enabled by the fact that *E. coli* utilized the native promoters and ribosomal binding sites found in the andrimid BGC, which allowed the production of resistance genes. Annotation of the andrimid BGC noted the presence of 21 open reading frames (ORFs) involved in both biosynthesis and resistance. Two genes, *admQ* and *admT*, were predicted to confer resistance to andrimid as they encoded a major facilitator subfamily transporter and a *β*-subunit of ACC, respectively.[54] Expression of *admQ* in *E. coli* resulted in andrimid resistant clones. Subsequent investigations by Walsh and co-workers showed that expression of *admT* conferred andrimid resistance to sensitive *E. coli*, and subsequent *in vitro* assays revealed that the IC_{50} value of native *E. coli* ACC (AccA/AccD) increased from 12 nM to 500 nM when AdmT replaced AccD, a 41-fold increase. This increase may be an underestimation as the *E. coli* strain used to overexpress the AccA/AdmT protein complex contains a native *accD* gene, which may have resulted in mixed populations of AccA/AdmT and AccA/AccD.[55] Comparison of the sequences of AccD and AdmT suggested that position 203 (*Escherichia coli* numbering), found in the active site, was a major contributor to andrimid resistance. Exchange of the methionine residue found in AccD for a leucine residue (found in the corresponding position of AdmT) resulted in an increase in IC_{50} calculated for *in vitro* activity from 12 to 55 nM.

Interestingly, earlier work to identify the mechanism of action (MoA) of andrimid using an analog (**10**) identified a resistant *E. coli* mutant that upon sequencing of the *accD* locus showed a mutation that results in the substitution of a serine residue with tyrosine (position 207, *E. coli* numbering, Figure 2). In contrast, mutants derived from exposure of *Staphylococcus aureus* and *Bacillus subtilis* to **10** led to mutations being observed in *accA*.[53] This demonstrates that self-resistant protein variants represent one possible path of evolution and that perhaps native resistance genes might inform researchers as to what issues may appear in the clinic.

Mycophenolic Acid. Mycophenolic acid (MPA; **11**) is an FDA approved immunosuppressive drug produced by *Penicillium* species and exerts its effects through the inhibition of inosine-5′-monophosphate dehydrogenase (IMPDH). IMPDH is an enzyme that catalyzes the conversion of IMP to xanthosine-5′-monophosphate (XMP), which is the rate limiting step in guanine biosynthesis and is necessary for the growth of both T and B cells.[56] To identify the mycophenolic acid BGC in *Penicillium brevicompactum*, Hertweck and co-workers used self-resistance as a guide. PCR cloning of IMPDH-encoding genes revealed that at least two copies were present in the genome. These PCR products were used to screen a cosmid library, which led to the isolation and sequencing of a cosmid containing the putative MPA biosynthetic genes. In this BGC, the gene *mpaF* was identified as an IMPDH-encoding gene and predicted to be involved in MPA tolerance. Phylogenetic analysis of MpaF demonstrated that *mpaF* was most likely obtained by horizontal gene transfer as it was most closely related to *Penicillium chrysogenum mpaF* and not the housekeeping copy found in the genome of *P. brevicompactum*.[57] A parallel genome mining approach noted that *Penicillium* strains contained two copies of genes encoding IMPDH (named IMPDH-A and IMPDH-B (MpaF-homologue)), although not all *Penicillium* strains that encoded two IMPDH homologues produced MPA.[58] Interestingly, phylogenetic analysis of 12 IMPDH proteins (two each from six strains of *Penicillium*) seemed to indicate that the resistant IMPDH may have arisen through gene duplication, most likely at an early stage of diversification of *Penicillium* subgenus *Penicillium*. Exchange of *imdA* in the heterologous host *Aspergillus nidulans* with *mpaF* resulted in a substantial increase in MPA resistance.[58] *In vitro* characterization of IMPDH-A and IMPDH-B from *P. brevicompactum* (*Pb*IMPDH-A and *Pb*IMPDH-B, respectively) showed that the two proteins have a

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different mechanism of inhibition compared to IMPDH from *A. nidulans* (*An*IMPDH). Inhibition of *An*IMPDH was best described by uncompetitive inhibition of both IMP and NAD^+ as well as a lower IC_{50} value (26 ± 2 nM). *Pb*IMPDH-A had a naturally occurring resistance, as shown by the increased IC_{50} value (430 ± 30 nM) but also inhibition data best fit with a noncompetitive/mixed pattern of inhibition against IMP. This was partially explained by the fact that both *Pb*IMPDH-A and *Pb*IMPDH-B produced less of the enzyme bound reaction intermediate. One wonders if this change in enzyme properties "primed" the ancestral IMPDH in *Penicillium* strains for evolution of greater MPA resistance.[59]

Platensimycin/Platencin (12/13). Platensimycin and platencin are structurally related antibiotics that exert their antimicrobial activity through inhibition of fatty acid biosynthesis, with both inhibiting FabF/B and platencin inhibiting FabH. These terpene-containing natural products were identified by researchers at Merck and Co. using an antisense-induced sensitivity screen.[60,61] Identification of the BGCs responsible for antibiotic production from *Str. platensis* MA7327 and MA7339 was accomplished by the Shen group.[62] During annotation of the BGC, they noted the presence of four genes (*ptmP1−4*/*ptnP1−4*) that were hypothesized to be involved in self-resistance due to amino acid similarity to *β*-ketoacyl−acyl carrier protein (ACP) synthases (KAS) III condensing enzymes (*N*-terminus of PtmP1/PtnP1), acetyl-CoA acetyltransferases (PtmP2/PtnP2), KAS II condensing enzymes (PtmP3/PtnP3), and major facilitator superfamily proteins (PtmP4/PtnP4). Subsequent experiments revealed that heterologous expression of *ptmP3* resulted in an increased resistance for both platensimycin (MIC increased from 64 μg/mL to >256 μg/mL) and platencin (MIC increased from 0.125 μg/mL to >64 μg/mL) in the sensitive strain *Str. albus* J1074.[63] Overexpression of the other genes did not result in an increased MIC being observed. As PtmP3/PtnP3 were similar to KAS II condensing enzymes, the Shen group postulated two hypotheses about the role of PtmP3. First, PtmP3 could act as a decoy target, sequestering platensimycin or platencin away from FabF. Second, PtmP3 was a fully catalytic enzyme that participated in fatty acid biosynthesis. To differentiate between these two possibilities, the active site cysteine residue was substituted with a leucine residue (PtmP3-C162L). This amino acid substitution returned the MIC to the wild-type levels when the protein was heterologously expressed in *Str. albus* J1074. Interestingly when *ptmP3* was deleted from the genome of *Str. platensis* MA7327 (simultaneously with *ptmU4*, *ptmA3*, and *ptmP4*), the resulting strain *Str. platensis* SB12021 was sensitive to platencin but retained resistance to platensimycin. This intrinsic resistance to platenimycin was traced to *fabF* identified at a disparate genomic locus.

Indolmycin (14). Indolmycin is an antibiotic produced by *Str. griseus* ATCC 12648[64] and exerts its biological activity through the inhibition of trptophanyl-tRNA synthetase (TrpRS). In identifying the indolmycin BGC, Ryan and co-workers searched a draft genome and identified three genes encoding putative TrpRS enzymes. One, subsequently named *ind0*, was located in a 10.3 kb BGC that consisted of nine genes with *ind0* oriented on the opposite DNA strand compared to the other eight genes (Figure 3A). This BGC was confirmed to be responsible for biosynthesis of indolmycin through a combination of genetic knockout mutants and *in vitro* reconstitution.[65] Interestingly, *ind0* was not amplified by oligonucleotides targeting the indolmycin-resistance gene previously identified in *Str. griseus* NBRC 13350,[66] SGR3809. This third TrpRS was located at a

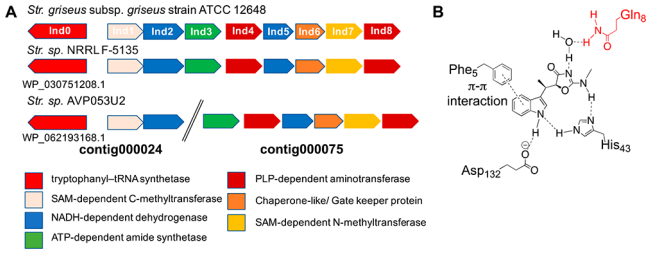

Figure 3. Indolmycin BGCs and interaction of indolmycin and TrpRS. (a) Predicted indolmycin BGCs identified through genome mining using Ind0 as a query sequence. Ind0 (and homologues) are colored in red. IndX, where X = 1−8, are based on the naming proposed by Ryan and co-workers. (b) Interactions contributing to the binding of indolmycin to the TrpRS from *Bacillus stearothermophilus* (PDB 5DK4). The equivalent position to Gln8 (*B. stearothermophilus* numbering) is substituted by a Thr residue in Ind0 and similar proteins (Supporting Information Figure 2). Hydrogen bonds are indicated by dashed lines.

separate genetic locus of *Str. griseus* ATCC 12648. This shows a potential challenge with genome mining based on self-resistance as self-resistance genes are not absolutely conserved in their proximity to the biosynthetic genes. Previous bioinformatic analysis of SGR3809 showed that it was quite distinct from TrpRSs identified in other *Streptomyces* strains with a sequence similarity of 38% to that of TrpRS1 (aka SCO3334), previously identified as an indolmycin resistant TrpRS from *Str. coelicolor* A3(2).[66,67] However, this phylogenetic analysis was performed before bacterial genome sequencing was commonplace, and only a few *Streptomyces* species genomes were available. Performing the phylogenetic analysis anew using 154 *Streptomyces* TrpRS sequences (Top 50 BLAST hits from SCO3334, SCO4839, Ind0) shows that Ind0 and SGR3809 are only moderately related, and both SGR3809 and Ind0 are more distantly related to TrpRS1 (Supporting Information Figure 1). Examining the genetic context around the four TrpRS encoding genes that cluster with Ind0, we could identify an indolmcyin BGC in the two most closely related sequences from *Streptomyces sp.* AVP053U2 (WP_062193168.1) and *Streptomyces sp.* NRRL F-5135 (WP_030751208.1). A full indolmycin BGC was identified in *Streptomyces sp.* NRRL F-5135, demonstrating the potential of mining by self-resistance to identify hard to find clusters (Figure 3A). In contrast, *Streptomyces sp.* AVP053U2 genes encoding Ind0, Ind1, and Ind2 were identified on contig000024. BLAST analysis identified the genes encoding Ind4−Ind8 on contig000075, which provides an example where an incomplete genome can hamper the search for natural product BGCs. In *Streptomyces* sp. NRRL F-3213 (WP_037816636.1) and *Streptomyces* sp. AA4 (WP_009083072.1), the TrpRS encoding gene is not located in a putative natural product BGC (data not shown). It is possible that the *ind0* genes for the latter two *Streptomyces* were acquired through horizontal gene transfer in response to environmental exposure to indolmycin as both *Streptomyces* sp. NRRL F-3213 and *Streptomyces* sp. AA4 contain a second copy encoding a TrpRS. A glutamine residue found in *Bacillus stearothermophilus* TrpRS (Gln8, *B. stearothermophilus* numbering) interacts with the nitrogen in the 3-oxazolin-4-one ring of indomycin indirectly through a water molecule (Figure 3B, 3.5 Å N−N distance). This residue is substituted with a threonine residue in Ind0. While currently untested, this substitution could possibly contribute to a lower binding affinity of indolmycin to Ind0 as this residue is involved in stabilizing the inhibited indolmycin−TrpRS complex.[68] This same substitution (Gln8Thr)

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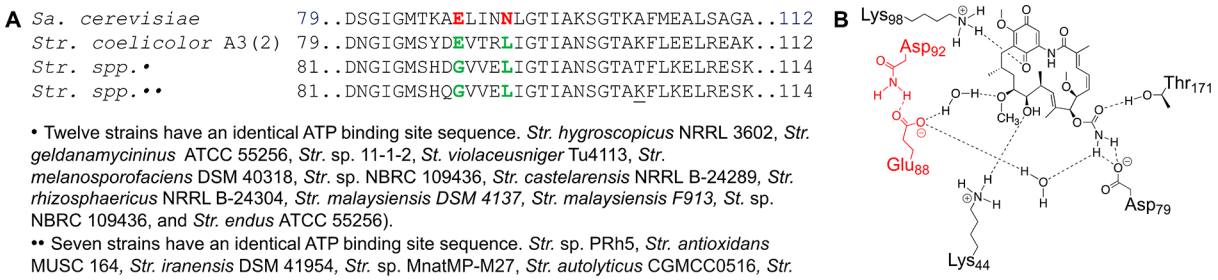

Figure 4. Binding of geldanamycin (**15**) to HSP90. (a) Sequence alignments of the ATP binding pocket HSP90/HtpG from *Sa. cerevisiae* and *Streptomyces* strains. Amino acids highlighted in red were found to interact with the methoxy group present in **15**. (b) Schematic diagram of residues involved in binding of **15** to *Sc*HSP90 identified from PDB 1A4H. Substitution of the amino acids shown in red reduces the binding affinity of **15** to HSP90. Hydrogen bonds are indicated by dashed lines.

is conserved in the four similar TrpRS's discussed above (WP_062193168.1, WP_030751208.1, WP_037816636.1, WP_009083072.1). Due to the differences in primary sequence of Ind0 and other TrpRS protein sequences (Supporting Information Figures 1 and 2), it is likely that other residues play a role as well.

Geldanamycin (15). Geldanamycin (GdA) is a naturally occurring heat shock protein (Hsp90) inhibitor that has served as a lead compound for the development of anticancer agents. It was first identified from cultures of *Streptomyces hygroscopicus var. geldanus var. nova* and characterized as having broad antibiotic acitivities,[69] although its interaction and inhibition of an HSP90-pp60v-src complex was not shown until 24 years later.[70] The Hsp90 protein is essential for eukaryotic survival, and the HSP90 homologue, HtpG, is essential for prokaryotic survival. Due to the highly conserved nature of Hp90/HtpG, it was reasoned that the producing strain would produce a variant of HtpG that was defective when it came to binding of geldanamycin. Cloning of the *Str. hygroscopicus htpG* locus revealed two points of discussion. First, there was only one copy of *htpG* in the genome, and it was not found in proximity to the geldanamycin BGC, as in the cases described above. Second, the lone *htpG* encoded a HtpG variant with two amino acid substitutions in the active site. These amino acid substitutions occur within the ATP binding pocket and are E88G and N92L (Figure 4A, *Saccharomyces cerevisiae* numbering).[71] Both substitutions are located on the A face of the ATP binding pocket, the region in which the C-12 methoxy group of GdA is found. Introduction of these substitutions together into the equivalent positions of the yeast (*Sa. cerevisiae*) Hsp90 protein led to a 10-fold decrease in binding affinity for GdA, while individual introduction of these substitutions led to no change in binding affinity for GdA.[71] The presence of a leucine residue at position 92 in *Str. coelicolor* (and other strains, data not shown) suggests that *Streptomyces* strains were situated to evolve resistance to GdA through the acquisition of one amino acid substitution.

USE IN DETERMINING THE MOA OF NATURAL PRODUCTS

DnaN. One of the prominent ways that self-resistance has been taken advantage of during genome mining is assigning a mechanism of action to a biologically active compound. In these cases, the compound is identified through traditional, bioactivity guided fractionation and structure determination, and the BGC is subsequently identified. One recent example was during a recent reinvestigation of the griselimycins as anti-*Mycobacterium tuberculosis* agents. It was suggested that griselimycin (**16**) had a different mechanism of action than currently used tuberculosis drugs as **16** is equally active against both drug-susceptible and drug-resistant *M. tuberculosis* strains.[72] The producing strain, *Str.* sp. DSM 40835, was sequenced, and during annotation of the griselimycin BGC a gene, *griR*, encoding a putative self-resistance variant of DnaN was identified. GriR was the second copy encoded in the genome as a housekeeping copy (*dnaN*) with 51% identity was identified. Introduction of *griR* into *Str. coelicolor* resulted in resistance to griselimycin. Investigation of the spontaneously resistant strains of *Mycobacterium smegmatis* revealed that the resistance was derived from duplication of a chromosomal segment that encodes *dnaN*.[72] This means that spontaneous resistance does not require mutation in *dnaN* but that overproduction of DnaN can act as a compound sponge, sequestering griselimycin. This demonstrates that just because a self-resistant protein variant is found in a producing organism it does not mean that this is the pathway of resistance that will be observed to arise from spontaneous mutations.

Pentapeptide Repeat Proteins. The cystobactamids (**17**) were isolated from the myxobacterium *Cystobacter* sp. by the Müller group and showed broad range antibacterial activity against both Gram-positive and Gram-negative bacteria.[73] Structure determination showed that the cystobactamids contained *p*-aminobenzoic acid subunits. However, the mechanism of action was unknown as the cystobactamids were discovered using a whole cell assay. During the sequencing of the biosynthetic gene cluster, an open reading frame, *cysO*, was identified and postulated as a self-resistance protein. CysO shows similarity to proteins in the pentapeptide repeat protein family that had previously been characterized as resistance determinants of DNA topoisomerase inhibitors.[74−76] *In vitro* biochemical testing showed that the cystobactamids did indeed inhibit both *E. coli* DNA gyrase and topoisomerase IV with IC_{50} values ranging from 0.26 ± 0.06 to 89.5 ± 0.8 μM.

These two examples illustrate how instrumental self-resistance proteins can be in identifying the mechanism of action for natural products identified during whole cell assays.

Targeted Genome Mining. Recently, the Moore group used self-resistance proteins as a guide to identify an orphan biosynthetic gene cluster. Heterologous expression revealed that this BGC encoded for the thiotetroamide class of antibiotics (**18**).[77] The identification of a polyketide synthase containing BGC that encoded a homologue of FabB/F subsequently named *ttmE/ttmJ* followed by heterologous expression led to the production of the thiotetroamides (**18**). TtmE had high similarity to PtmP3 (65%/79%, identity/similarity) and PtnP3 (65%/79%, identity/similarity), which had previously been shown to serve as self-resistance determinants in platensimycin (**12**) and platencin

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(**13**) producers, respectively.[63] Interestingly, the deletion of *ttmE* and *ttmJ* individually or in combination did not decrease the production of the thiotetroamide natural products in the heterologous host *Str. coelicolor*. The authors speculate that this may be due to the low compound titers achieved through heterologous expression. The deletion of *ttmJ* produced a strain more sensitive to exogenously applied thiotetroamide C (**17**, a). Despite the similarity to PtmP3/PtnP3, cross-resistance to platensimycin was not addressed in this study.

False Positives. Inhibitors of tRNA synthetases are found among natural products including indolomycin (described above). However, amino acyl-loaded tRNAs have been identified as biosynthetic intermediates as covered in the recent review by Ulrich and van der Donk.[78] This suggests that the presence of tRNA synthetases in natural product BGCs could lead to mistaken identification of the mechanism of action. One example is the BGC responsible for the production of valanimycin (**19**).[79] While the biosynthesis has not been completely elucidated, one of the key steps involves the activation of serine by VlmL (Ser-tRNA synthetase) to Ser-tRNA, which is then utilized as an activated electrophile for coupling with isobutylhydroxylamine through the action of VlmA.[80] Of note is that VlmL has 39% identity/55% similarity at the amino acid level to SerRS of *Streptomyces coelicolor* A3(2),[81] a similar level of identity to Ind0 from the indolmycin cluster to SCO4839 from *Str. coelicolor* (44/58% identity).[65] This might lead one to believe that valanimycin is an inhibitor of Ser-tRNA synthetases in analogy to the case of indolmycin described above. How to differentiate these scenarios is a question for future research.

In our own research, we have identified that type II PKS clusters have a high probability of containing a gene encoding an AccD homologue. We also noted that this gene is consistently upstream of a gene encoding a biotin-(acetyl-CoA-carboxylase) ligase homologue, suggesting these AccD homologues are responsible for the supply of malonyl-CoA (or other precursor molecule). We have written code that looks for homologues of biotin-(acetyl-CoA-carboxylase) ligase in the surrounding four ORFs (two upstream and two downstream). If a homologue of biotin-(acetyl-CoA-carboxylase) ligase is detected, the cluster is flagged as a false positive. One interesting question that has come out of this observation is why only some of the annotated type II PKSs contain the genes for biosynthesis of malonyl-CoA. This is currently under investigation in our lab.

False Negatives. One notable exception to the co-occurrence of both sensitive and resistant drug target homologues involves the RNA polymerase inhibitor rifamycin B (**20**), produced by *Amycolatopsis mediterranei* S699. As predicted, a gene that encodes for RpoB (β-subunit of RNA polymerase) is found in proximity to the rifamycin BGC identified in *A. mediterranei* S699. Unexpectedly, no other homologues of RpoB are encoded in the *A. mediterranei* S699 genome.[82] Multiple sequence alignments and phylogenetic analyses can also be employed to determine if putative self-resistant protein variants have been acquired *via* horizontal gene transfer (HGT), or if amino acid substitutions are present in the active site that are absent in other closely related homologues, as was found during the analysis of RpoB from rifamycin-producing strains of *Salinispora* spp. Differences between the species phylogeny and *rpoB* phylogeny suggested that *rpoB* was partially acquired by HGT as self-resistance against rifamycin.[83]

An additional example is the previously mentioned HSP90 inhibitors. Previous research has shown that the heat shock protein 90 homologues from each of the producing organisms (HtpG from *Streptomyces hygroscopicus*; *Sh*HtpG) and HSP90 (from *Humicola fuscoatra*; *Hf*HSP90) contain amino acid substitutions, which confer self-resistance to the produced natural product.[71,84] This illustrates that not all self-resistance protein variants are duplicated, and future work is necessary to develop methods to identify these false negatives.

POTENTIAL OF SELF-RESISTANCE IN THE DISCOVERY OF NEW NATURAL PRODUCTS

Prioritization of Gene Clusters. The vast amount of genome data that has been generated has revealed that many bacterial strains harbor more BGCs than molecules observed in laboratory culture. These orphan BGCs are thought to provide a reservoir of untapped biosynthetic potential. The question of how to prioritize resources and which BGCs to investigate has now become a major focus with algorithms that cluster BGCs,[12−14] algorithms that compare the predicted products to known molecules,[15] and algorithms clustering molecules and mechanisms of action.[16] Recently, a web-based tool named the Antibiotic Resistant Target Seeker (ARTS) has been released to help prioritize microbial strains by identifying those with the potential to produce a natural product with a desired mechanism of action.[18] ARTS utilizes the antiSMASH platform to identify biosynthetic gene clusters and then locates genes identified as core genes (those found in a majority of strains from a given microbial family) or known resistance genes and assigns these genes a "proximity score," which helps determine if they are truly part of the BGC. These results can then be examined by the user to prioritize the BGCs that they wish to pursue. Currently, ARTS is optimized for actinomycete analysis, but future work will expand its usefulness to other microbial families. A benefit of ARTS is that, when automated, sequenced genomes can be screened for multiple desired self-resistance protein variants simultaneously. The most prominent negative feature of ARTS is that it is restricted to the BGCs currently identified by the antiSMASH platform.

As an example of how genome mining can be guided and assisted by self-resistance, we surveyed the HtpG homologues found in *Streptomyces* strains that have their genomes deposited in the public NCBI database. We examined the HtpG proteins for the amino acid substitutions found in *Str. hygroscopicus* NRRL 3602. In searching the NCBI database, we identified 19 strains of *Streptomyces* that contained an *htpG* gene encoding an HtpG protein with E88G and N92L substitutions (Figure 4), which was shown to be important to self-resistance to geldanamycin. Each strain was then analyzed by antiSMASH[85] and by manually searching the NCBI genome record. We looked for the presence of BGCs that could produce the known HSP90 inhibitors **13** or herbimycin. Of the 20 strains identified, five contained a full geldanamycin BGC (*Str. hydroscopicus* NRRL 3602 (known geldanamycin producer), *Str. autolyticus* CGMCC0516, *Str.* sp. strain 11−1−2, *Str. malaysiensis* DSM 4137, and *Str. malaysiensis* F913; Figure 5A). An additional five strains have a partial BGC with identity to the geldanamycin BGC (*Str.* sp. SPMA113, *Str.* sp. NBRC 109436, *Str.* sp. PRh5, *Str. endus* ATCC 55256, and *Str.* MnatMP-M27). In addition, two strains had a complete herbimycin BGC (*Str. hygroscopicus* AM 3672 (known herbimycin producer) and *Str. violaceusniger* Tu 4113), while *Str. rhizosphaericus* NRRL B-24304 contained a partial herbimycin BGC (Figure 5B). Two strains (*Str. geldanamycinicus* ATCC 55256 and *Str. antioxidans* MUSC 164) contain neither a geldanamycin nor a herbimycin BGC even though *Str. geldanamycinicus* ATCC 55256 is a known producer of 4,5-dihydrogeldanamycin,[86]

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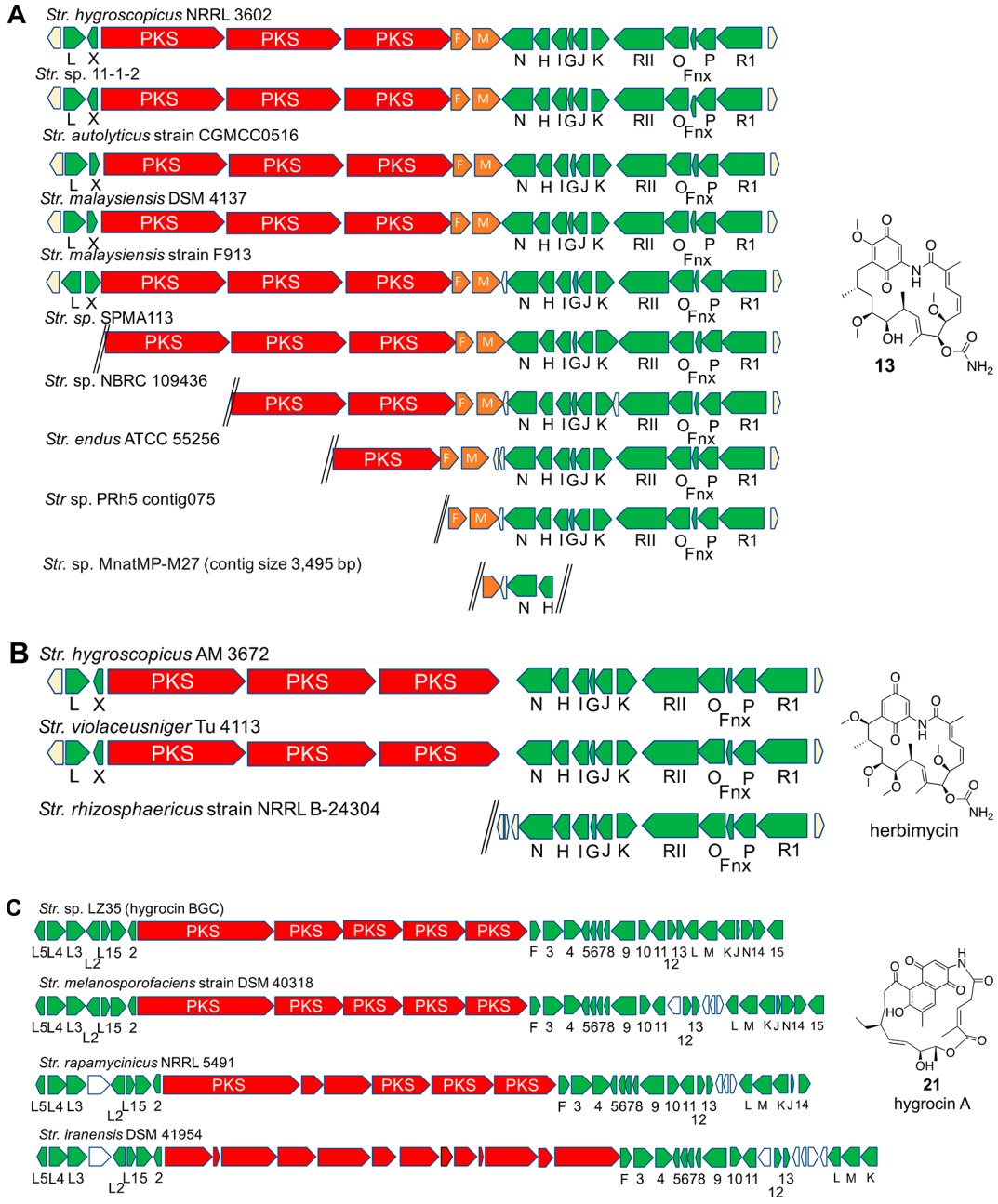

Figure 5. Predicted full and partial geldanamycin (panel A), herbimycin (panel B), and hygrocin (panel C) BGCs identified through genome mining of *Streptomyces* strains that contained amino acid substitutions in HtpG that confer resistance to known HtpG/HSP90 inhibitors. Polyketide synthase encoding genes are colored in red; other biosynthetic genes are colored green, while genes outside the defined boundaries of the BGC are colored light yellow, and genes of unknown function are filled white.

while *Str. castelarensis* NRRL B-24289 had many small gene clusters annotated during AntiSMASH analysis (89 BGCs identified), which made the presence of a geldanamycin or herbimycin BGC difficult to ascertain. This provides an example where self-resistance guided genome mining opens up the possibility of deprioritizing certain strains, as growth and fermentation of these strains is likely to lead to the rediscovery of geldanamycin or herbimycin.

The three remaining strains did not contain a herbimycin or geldanamycin BGC (*Str. iranensis* DSM 41954, *Streptomyces melanosporofaciens* strain DSM 40318, and *Str. rapamycinicus* NRRL 5491). Closer inspection of these genomes revealed that all had a BGC with similarity to the known BGC responsible for production of the known ansamycin, hygrocin (**21**; Figure 5C). Because of the amino acid substitutions identified in HtpG, we propose that hygrocin exerts its cytotoxic bioactivity[87] through inhibition of HSP90, providing another example of how self-resistance protein variants can aid in the identification of MoA for natural products.

Enrichment of Producers. One recent example of utilizing self-resistance to enrich producers of molecules with specific examples comes from the Wright laboratory.[17] Here, soil samples were cultivated on antibiotics (vancomycin and rifamycin). The hypothesis being that organisms that were capable of growth in the presence of inhibitors of cell wall biosynthesis (vancomycin) or RNA polymerase inhibitors (rifamycin) would have a greater chance of producing molecules with the same mechanism of action. From 100 random environmental isolates that grew in the

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presence of vancomycin, the Wright group was able to isolate the previously unknown sulfated glycopeptide antibiotic, which they named pekiskomycin (**22**). This demonstrates the power of preselecting environmental isolates as the percentage of potential glycopeptide antibiotic producers isolated in this fashion (11%) was much greater than that observed by randomly screening 1000 previously collected isolates (0.1%). In contrast, exposure to rifamycin (20 μg/mL) did not result in only rifamycin or rifamycin-like producing strains being isolated but did enrich environmental samples for ansamycin producing strains, and the researchers were able to identify a geldanamycin producer. However, it is to be noted that one of the biggest hurdles in this research was identifying conditions that produced the desired active molecule, and it may be most effective if this enrichment technique is used upstream of genome sequencing and targeted BGC upregulation.[9] One drawback of this approach is that the enrichments can only be performed with compounds that have been isolated and is thereby limited to known pathways and antibiotics that can be obtained in sufficient quantity.

Potential in Plants. Previous research into the anticancer alkaloid camptothecin (**23**) producing plants *Camptotheca acuminata*, *Ophiorrhiza pumila*, and *O. liukiuensis* revealed that these plants contained amino acid substitutions in their DNA topoisomerase I that confers resistance to camptothecin.[88] These results suggest that self-resistance could be used to screen plant material prior to large scale harvesting and extraction to determine which species possess the highest probability of producing a compound with an inhibitory activity toward a desired target; however, very little work in this area has been done.

Animals have also been shown to invoke self-resistant protein variants as a mechanism for combating self-toxicity. The most studied case is that of resistance to voltage-gated sodium channel inhibitors (*e.g.*, tetrodotoxin (**24**), TTX) in amphibians and even predatory snakes. We propose that bioprospecting could start with DNA/RNA isolation from collected specimens to determine if they had a high potential to produce a molecule with a desired bioactivity followed by genome sequencing and genome mining to identify the genes involved in the production of secondary metabolites. This might alleviate the concerns of harvesting large quantities of endogenous flora and fauna from remote locales but requires that genome sequencing, assembly, and genome mining in eukaryotes be significantly streamlined and improved.

FUTURE DIRECTIONS

How self-resistance is used to help natural products researchers remains to be fully explored. Programs like ARTS, large scale cluster comparisons and binning,[12−14] and biosynthetic approaches will help guide research into microbial natural products and help prioritization. Currently, we estimate that the rate of occurrence of self-resistant protein variants is between 1 and 5% of currently sequenced *Streptomyces* genomes. We consider this skewed as most genomes were sequenced to identify gene clusters from isolated bioactive natural products. As more genomes are randomly sequenced, it will be interesting to determine whether the frequency of self-resistant protein variants increases or decreases. It also remains to be determined whether self-resistance can help in prioritizing natural product discovery from plants and other macro-organisms as discussed above. One question is how eukaryotic organisms that harbor endosymbionts avoid toxicity from natural products produced by these same endosymbionts. One could envision many different mechanisms of resistance from chemical modification as was recently proposed for the mandelalides,[89] subcellular sequestration of the endosymbionts and natural products to self-resistant protein variants. It is also possible that the same eukaryote utilizes multiple methods of resistance. More in-depth investigations are needed in this area.

Problems to Overcome. In addition to the challenges described above, one other major hurdle exists for genome mining. Genome mining is dependent on the quality of the genome sequences deposited into the public databases. In recent years, the explosion in genomic data has been a boon to genome mining, and the reduced cost for acquiring a draft genome has plummeted to approximately $1,000 for a bacterial genome. However, the cost to complete a genome remains high and the time investment significant. This means that now most of the genomic data are being deposited in the draft stage. This represents a problem on two fronts for genome mining. First, the positions of BGCs in the genome are not truly known, so large scale comparative genomics and evolution of BGC acquisition are difficult to investigate directly. Second, and more important to natural products discovery, is that BGCs can be fragmented and incomplete in the deposited genome data. This is in part due to the repetitive, modular nature of NRPS and PKS genes/proteins, which sometimes makes it difficult to assemble complete BGCs from short reads.

We believe that some of these issues, including incomplete genomes and BGCs, will be overcome with the newer third generation sequencing technologies such as the PacBio system, which can give reads up to 10 000 bases as noted by others.[90] But care in data analysis is now more important than ever because reviewers and editors may not be able to examine the genomic data, particularly in large sequencing efforts.

One item that was briefly mentioned but should be expanded on is the fact that searching by known self-resistance mechanisms might skew genome mining results to known compounds or structural analogs. This can be seen in the example of pekiskomycin isolation,[17] and the examples given here for indolmycin and the geldanamycin/herbamycin/hygrocin BGCs. Although the work that resulted in the identification of the thiotetroamide antibiotic BGCs[77] suggests that even looking at known pathways of resistance can result in the discovery of new compounds. This should encourage researchers to couple self-resistance genome mining with BGC comparisons to dereplicate known classes of compounds and to explore "new" mechanisms of resistance.

CONCLUSION

Here, we review some recent literature pertaining to self-resistance in natural product producers. We have included ways that self-resistance has been used to enhance natural products research from prioritizing microbial strains (both bacteria and fungi) to identifying the mechanism of action of natural products. We have also attempted to point out some future areas of research and deficiencies in knowledge/techniques that need to be answered to enhance the contribution to genome mining for new natural products. We propose that self-resistant protein variants could be a useful tool for genome mining of natural products that will help us understand how microbes (and possibly plants) have evolved the ability to produce natural products.

ASSOCIATED CONTENT

Supporting Information

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KEYWORDS

Biosynthetic gene cluster (BGC): a collection of genes that are colocalized in a microbial genome and coregulated to produce a compound

IC_{50}: the concentration of a compound where 50% of the maximal inhibitory activity is observed

Mechanism of Action (MoA): the particular pathway that a compound inhibits to produce its pharmacological effects

Natural products: small molecular weight compounds (typically less than 2000 Da) produced by organisms

Nonribosomal peptide synthetase (NRPS): large multimodular protein complexes that are responsible for the assembly of peptide natural products. Each module contains domains for the selection, activation, tethering, condensation, and modification of a precursor molecule, typically a proteinogenic or nonproteinogenic amino acid into a natural product.

Polyketide synthase (PKS): large multimodular protein complexes that are responsible for the assembly of polyketide natural products. Each module contains domains for the selection, activation, tethering, condensation, and modification of a precursor molecule, typically malonyl-CoA or methylmalonyl-CoA into a natural product.

Secondary metabolite: an alternate term for natural product.

Self-resistance protein variants: proteins that have evolved amino acid substitutions to prevent the binding of a toxic natural product but remain catalytically active.

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