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Genome mining and prospects for antibiotic discovery

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Natural products are a rich source of bioactive compounds that have been used successfully in the areas of human health from infectious disease to cancer; however, traditional fermentation-based screening has provided diminishing returns over the last 20–30 years. Solutions to the unmet need of resistant bacterial infection are critically required. Technological advances in high-throughput genomic sequencing, coupled with ever-decreasing cost, are now presenting a unique opportunity for the reinvigoration of natural product discovery. Bioinformatic methods can predict the propensity of a microbial strain to produce molecules with novel chemical structures that could have new mechanisms of action in bacterial growth inhibition. This review highlights how this potential can be harnessed; with a focus on engineering the expression of silent biosynthetic gene clusters predicted to encode novel antibiotics.

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Introduction

During the Golden age of Antibiotic Discovery numerous life-saving medicines were developed from the natural products (NPs) produced by microbes and particularly the Actinobacteria. However, the returns from traditional fermentation-based screening diminished over time, rediscovery became rife, and the pipeline for NP antibiotics dried up [1]. The rise of extensive and ubiquitous antibiotic resistance, coupled with the decline in the discovery and development of novel therapies, has led to the so-called 'Antibiotic Crisis', and prompted the WHO to declare this one of the greatest threats to human health [2]. New antibiotics, in addition to other therapeutic options for infectious disease, are desperately needed. Critically, new mechanisms of action and novel chemical classes will likely be required to overcome existing mechanisms of clinical resistance.

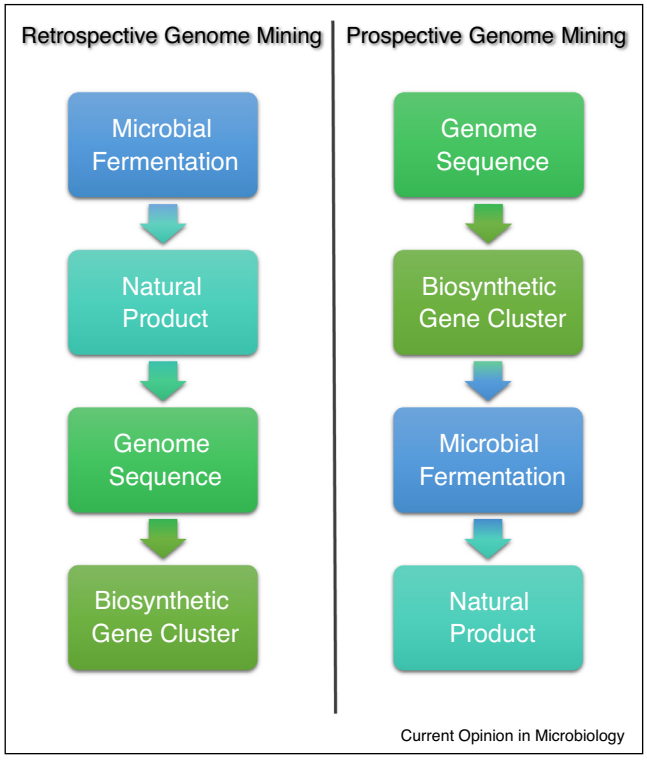
NPs are synthesized by dedicated enzymes, which are usually encoded by genes co-located in a region of the bacterial genome known as a biosynthetic gene cluster (BGC). In the age of high-throughput genome sequencing, it has become apparent that microbial genomes can encode large numbers of BGCs, suggesting that they have the potential to produce many more NPs than have been identified through conventional fermentation-based screening [1]. The assignment of known NPs to the BGCs that encode their production has enabled the determination of the 'rules' governing their synthesis ('Retrospective Genome Mining'; Figure 1). In turn, this has facilitated the development of BGC predictive tools such as antiSMASH [3] and PRISM [4], and the establishment of BGC databases such as MiBiG [5]. Using 'Prospective Genome Mining' (Figure 1), DNA sequences alone can now be used to predict the biosynthesis of an NP, its class and, in many cases, the final structure [1,6]. Realizing the potential of such BGCs to produce novel antibiotics has been an active area of research during the period 2017–18 (Table 1) and is the subject of this review.

Prospecting for new antibiotics

The toolbox of approaches that are now routinely used for the identification of novel BCGs, from identifying key microbial sources, high-quality genome data, including long-contiguity sequencing technologies such as PacBio and Oxford Nanopore, and the use of bioinformatic tools to infer novelty, have been extensively described [6,7]. Such methods can provide a 'short list' of potentially novel BGCs for further study, for example through activity-based screening of the producer strains [8••]. The challenge is then how to identify, *in silico*, those BGCs that are most likely to encode molecules that function as antibiotics, without the requirement for, potentially large-scale, activity-based screening. One common method is to focus on finding novel members of known classes of antibiotics; a 'chemocentric' search. This can be achieved by looking for BGCs with shared biosynthetic features, but with enough differences that novel chemical structures can be predicted. For example, by identifying rifamycin-like BGCs, containing additional tailoring genes, chemically novel members of this class were identified, which exhibit a new mode of action and evade existing resistance mechanisms [9••]. Through the *in-silico* detection of non-ribosomal peptide enzymes predicted to utilize specific amino acid precursors, novel cationic peptides were isolated, from *Bacillus* sp., that exhibit potent Gram-negative inhibitory activity [10••].

Another methodology that has recently been used to predict antibiotic-encoding BGCs is a 'resistance-guided'

Figure 1



A comparison of retrospective and prospective genome mining.

search strategy. This approach draws on the observation that microbial producers of antibiotics must provide themselves with a means of self-protection, for example through the provision of transport proteins, molecule or target modifying enzymes, or decoy targets. By prospectively searching for BGCs with associated resistance genes, the possibility that the encoded molecule might be an antibiotic is increased [11,12••]. A new bioinformatic tool has been developed to enable such searches to be carried out. The 'Antibiotic Resistant Target Seeker (ARTS)' program relies on the observation that duplicated copies of essential housekeeping genes, associated with BGCs, can provide self-resistance [13•]. This approach not only guides researchers to possible antibiotic-encoding BGCs, but can also be used to predict the mode of action and possible target of the resulting molecule. These are key pieces of information when prioritizing the selection of novel mechanisms of activity and avoiding existing clinical resistance. Resistance-guided screens represent an emerging strategy to assign putative function to NPs *a priori*.

The expression challenge

It has been observed that BGCs encoding new NPs, those not previously identified through the traditional approach of screening fermentation broths, are likely to be expressed at very low levels or not at all under laboratory conditions [14]. The inability to detect the products of these so-called cryptic or silent BGCs could be due to transcriptional-control or translational-control, post-translational modification or through limiting precursor or co-factor availability. Additionally, the enrichment and identification of novel molecules, which could be expressed at very low levels, can present a bottleneck [15•]. Rapid improvements in the area of mass spectrometry and comparative LCMS have been made and continued research in this area will be critical to discovery of new antibiotics [6,16,17,18•].

In the genomic era, the identification of a novel BGC presents two possibilities; (1) to attempt to produce the encoded molecule in the native strain from which it was identified or (2) to clone the predicted BGC and express in a heterologous host. Genetic manipulation of native strains can be challenging and time consuming. In addition, detecting the production of new molecules, particularly those that might be expressed at low levels, can be challenging in a background in which other 'masking' NPs, including known antibiotics, are present. Increasingly, genome mining is facilitating the exploration of more varied and exotic sources of NPs [6]. These sources can include metagenomic libraries, from which the producing bacterium is not culturable, or bacterial strains that are not readily amenable to genetic manipulation. Heterologous expression can be used to overcome such challenges; the chosen host should be genetically tractable and cloning the BGC enables a multitude of techniques in synthetic biology. Heterologous hosts have been shown to produce molecules that are otherwise cryptic or silent in native strains [19,20•]. Furthermore, the use of a heterologous host presents a convenient methodology for detecting the presence of new NPs through a differential analysis between the host, with and without the BGC of interest and addresses the 'masking' challenge present in native strain analysis [20•,21••].

Heterologous expression, however, can have its limitations and may not be successful in all cases or in every host tried [22–25]. It is also likely that such failures are underreported in the literature, preventing the community from learning from these examples. It has become apparent, over the last decade or so, during which heterologous expression has become a widely used approach, that host choice is a key factor in success and that the rules governing this are not well defined [22,24]. Several *Streptomyces* species have been harnessed as heterologous hosts and have been widely used with success in recent years [20•,22,26–31]. However, it is increasingly the case that BGCs are cloned from taxonomic groups other than *Streptomyces* sp. and questions remain about how well BGCs will express when transferred to a substantially different genus. There are recent examples of success in this regard, for example from *Actinoplanes* [32,33], *Frankia*

Table 1

Examples of novel antibiotics identified through prospective genome mining in the last two years. NRPS; non-ribosomal peptide synthase, PKS-I; type I polyketide synthase, PKS-II; type II polyketide synthase, CSR; cluster-specific regulator

Antibiotic	Molecule class	Mechanism of action	Spectrum	Method of identification	Expression system	Ref.
Brevicidine and laterocidine	NRPS, cationic peptide	Membrane disruption	Gram-negative, including colistin resistant *E. coli* (*mcr-1*)	Bioinformatic prediction of NRPS BGCs with features characteristic of cationic peptides	Native	[10••]
Pyxidilicyline	PKS-II	Topoisomerase IV	Gram-positive and efflux-incompetent *E. coli*	Resistance-guided BGC discovery and promoter replacement in native producer strain (myxobacteria)	Native and heterologus expression	[12••]
Malacidins	NRPS, cyclic lipopeptide	Lipid II binding (Ca-dependent)	Gram-positive with resistance to vancomycin and β-lactams	Culture-independent genome mining and heterologous expression	Heterologous expression	[21••]
Kanglemycins	PKS-I, ansamycin family	RNA polymerase; interfering with 5′-initiating substrate binding	Gram-positive with resistance to rifampicin, including *Mycobacterium tuberculosis*	Search for family-specific biosynthetic enzyme, AHBA synthase, with constraint of finding additional tailoring genes in BGC	Native	[9••]
Formicamycins	PKS-II	Not reported	Gram-positive with resistance to vancomycin and β-lactams	Survey of genomes of ant-associated actinomycetes with a focus on those strains with the highest number of BGCs	Native	[8••]
Auroramycin	PKS-I, polyene macrolactam	Not reported	Gram-positive with resistance to vancomycin and β-lactams	Activation of a novel PKS-I BGC by insertion of a constitutive promoter upstream of a CSR gene	Native	[58••]

[20•], and *Amycolatopsis* [27]. Development of Gram-negative bacterial species as hosts is also important since increasingly *Myxobacteria* sp., *Xenorhabdus* sp. and *Pseudomonas* sp. are emerging as talented producers of antibiotics [12••,14,23,34•].

Further considerations when using heterologous expression include clone to clone differences that can affect compound production levels, indicating that multiple clones should be assessed [27], and instability of large exogenous DNA fragments in host strains [28]. The spectrum of compounds produced in a heterologous host environment may not mirror precisely those in the native context [32,33] or could be entirely unexpected [35]. Finally, even when heterologous expression can be achieved, product yields could be significantly lower than in a native producer strain, if the metabolism or global regulatory system of the host does not match the requirements of the BGC [25].

Promoting identification of novel antibiotics

In the past two years, multiple approaches to solve the issues of low-level expression of NPs in both native and heterologous host systems have been advanced. These strategies form an important toolbox for unlocking the potential of novel antibiotic-encoding BGCs and will be described in the following sections. Examples where these tools have already been successfully employed in the production of novel antibiotics through prospective genome mining are highlighted in Table 1. In other cases, the examples given highlight potential areas of future promise for new antibiotic discovery.

Culture conditions

The use of elicitor chemicals, such as those produced through co-cultivation [36] or supplementation of growth media [37,38], can reprogram global regulatory pathways to induce silent BGC expression. The transient use of antibiotics to remodel primary metabolic flux into secondary metabolism can enhance NP production [39]. To address how many different culture conditions must be sampled to drive detectable NP production in a panel of strains, Crüsemann *et al.* used high-throughput extraction and mass spectrometry analysis of 26 marine isolates of *Streptomyces* sp. [40]. An increasing number of NPs were produced with each of three growth media tested. The

sequential extraction of fermentation broths with solvents of increasing polarity; ethyl acetate, *n*-butanol and methanol, resulted in each solvent contributing an increasing number of NPs. This analysis indicates that many different variables need to be screened when no prior knowledge about molecule production is available. This will require high-throughput fermentation, extraction and detection methods which may not be feasible in all circumstances. With many variables to test, the use of flask-based fermentation can rapidly become limiting. Many groups have now successfully adopted high-throughput growth formats in 24-well, 48-well or even 96-well microtiter plates [37,29,41]. A key factor in the cultivation of Actinomycetes is the morphology of the mycelium which can have differing effects on NP productivity. Wang *et al.* recently demonstrated that antibiotics such as thiostrepton and streptomycin can be used to alter the formation of 'pellets' and thus affect the resulting yields of NPs [42].

Manipulation of pleiotropic factors

NP biosynthesis is highly regulated in microbes; likely due to the energy input required to produce such complex molecules. Numerous mechanisms have been uncovered that serve as pleiotropic or global mechanisms of regulation of BGC gene expression. It has become increasingly apparent that, by manipulating these complex regulatory pathways, the expression of genetically silent BGCs can be induced. For example, a well-utilized approach has been the application of specific antibiotics to select for mutations, in either RNA polymerase or in certain ribosomal proteins, which can have pleiotropic effects on BGC expression [31,43,44]. To understand the types of pleiotropic changes that can influence NP production Zhang *et al.* interrogated the genome of an industrial strain of *Streptomyces albus*; the removal of competing biosynthetic pathways and insertions/deletions in key regulators were found to be major drivers of increased salinomycin production [45]. Another method that can be used to interrogate the role of pleiotropic factors in stimulating NP production is random transposon mutagenesis [46,47]. These studies have largely been focused, however, on improvement of production of known NPs and it is unclear how relevant these findings could be to induce the expression of cryptic antibiotic BGCs.

Proteins that act globally to influence NP production have been manipulated to activate silent BGCs, for example the pleiotropic regulators AdpA and AfsQ [48,49], and new pleotropic regulators are being uncovered such as MtrAB, which was found to influence antibiotic biosynthesis in two species of *Streptomyces* [50,51]. The crucial role of phosphopantetheinyl transferases (PPTases) in PKS/NRPS biosynthesis has previously been appreciated [31], although, only recently have these enzymes been harnessed as tools to induce the expression of cryptic BGCs for which PPTases might be limiting. The overexpression of genes encoding 'substrate promiscuous' PPTases, *sfp* and *svp*, in 33 actinomycete strains resulted in 70% displaying increased production of metabolites, including the induction of previously cryptic BGCs in two strains [52].

Key factors crucial for the production of specific NPs might be missing from a heterologous host, for example PPTases, MbtH proteins and other precursor-supplying enzymes. In addition to the previously well-characterized pptase, *sfp*, one of four PPTases in *Streptomyces avermitilis*, pptA2, was found to act promiscuously and to facilitate the heterologous expression of several PKS and NRPS compounds [53]. Supplementation of promiscuous PPTases, particularly if this translates to other host strains, could facilitate expression of cryptic clusters. Provision of limiting precursors might be achieved through the deletion of competing clusters in heterologous hosts [20•] or through the genetic manipulation of factors that can boost precursor supply [54,55]. Finally, for the expression of antibiotics, it is imperative that the producing strain is resistant to the produced molecule; this might be encoded within the BGC itself or could be intrinsic to the native producer. In the former case, it might be necessary to promote expression of the resistance genes from the BGC to ensure adequate compound production. In the latter case, either the resistance factor must be supplied in addition to the BGC or resistant forms of the strain should be selected [24].

Direct manipulation of gene expression from BGCs

Recent developments in CRISPR-Cas9 directed genetic manipulation may improve the prospects for activation of cryptic BGCs in native strains and the detection/identification of the produced compound through gene deletion. A recent improvement on the existing technology may allow additional bacterial strains, otherwise recalcitrant to manipulation, to be accessed [56]. A successful application of CRISPR-Cas9 technology, in a range of *Streptomyces* sp., was demonstrated through the incorporation of strong constitutive promoters upstream of biosynthetic operons in a variety of BGC types, which induced the expression of otherwise silent clusters [57]. Furthermore, this approach yielded a structurally novel Gram-positive active antibiotic from *Streptomyces roseosporus*, the product of a previously cryptic type I PKS BGC [58••]. Unfortunately, at the current time this technique is likely to be limited to BGCs with more simplistic operon organization and few intrinsic promoters, in addition not all native strains will be accessible with this approach.

Transcriptomics is another tool that has attracted attention in recent years. A study of four *Salinospora* strains, in conjunction with metabolomic analysis, indicated that as many as 72% of BGCs were transcriptionally active at levels that should enable metabolite detection. The authors argue that rather than gene expression being

the limiting factor in novel NP discovery, that extraction and analytical tools might be lacking. Furthermore, they suggest using transcriptional activity profiles to guide NP detection through mass spectrometry [15•].

The inadequate gene expression of cryptic BGCs in heterologous hosts has typically been suggested to be due to the lack of a key transcription factor or incompatibility in the timing of key gene transcription [31]. Several groups have recently focused on the direct manipulation of promoters within BGCs to eliminate this concern. Through enhanced BGC expression the production of novel congeners of known molecules was also promoted [22,24]. The correct balance of expression between different operons is important and driving hyper-transcription from each operon can be deleterious [24]. Several promoters have been described that can be utilized to control both the level and timing of gene expression, including a theophylline-dependent riboswitch and a *Streptomyces*-adapted T7 RNA polymerase system [22,59]. In addition, global transcriptional effects, mediated by localized chromosome structure, have been highlighted as a mechanism of BGC silencing and utilization of multiple integration sites revealed variances of up to eightfold in expression level [60].

Another important and recent focus has been on the role of translational control of expression in *Streptomyces* sp. The development of ribosome-occupancy mapping techniques has enabled the genome-wide profiling of translational efficiency and the determination of rules governing the selection of the most efficient ribosome binding sites [61]. Several promoter-RBS pairs have been developed that can drive high level expression of reporters such as GFP and GUS, and it remains to be seen how effective these might be at activating cryptic BGC expression [61,62].

Prospects for identifying new clinically relevant antibiotics

The combination of key improvements in the identification of novel BGCs through mining the genomes of increasingly diverse microbes, the expression of cryptic BGCs and the ability to detect those 'needle-in-a-haystack' new molecules, sets an exciting trajectory for the discovery of new antibiotics. In the last two years, there have been at least six novel antimicrobial molecules identified through prospective genome mining (Table 1). However, five out of the six newly identified molecules were active only against Gram-positive bacteria, while Gram-negative infection remains the greatest unmet medical need [2]. Although in-roads have been made into understanding the rules governing uptake across the Gram-negative envelope, this is far from a panacea, and converting such molecules to Gram-negative activity is likely to take a significant investment of medicinal chemistry innovation, time, and money [63,64••]. Focusing *a priori* on known classes that possess Gram-negative activity could be one beneficial approach [10••]. However, this could result in higher rates of cross-resistance with existing clinically used molecules and this should be tested as early as possible in the development process [9••,21••]. Resistance-guided searches will also be important for focusing on targets relevant to Gram-negative pathogens, although permeability and efflux concerns cannot be anticipated this way [12••]. Finally accessing new sources of novel BGCs, including Gram-negative producers or Gram-positive bacteria in competitive interactions with Gram-negative bacteria, is likely to yield dividends [34•].

Conflict of interest statement

The author is an employee and shareholder of Warp Drive Bio, a subsidiary of Revolution Medicines, Inc.

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Genome sequencing of ant-associated mutualistic actinomycetes revealed a 'talented' strain that encoded 39 BGCs. Through activity-based screening and isolation the authors identified novel, halogen-containing, molecules with activity against vancomycin and methicillin resistant*S. aureus*. This example illustrates how prospective genome

mining can be used to bias traditional activity-based screening towards the discovery of novel molecules.

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Using a chemocentric approach the authors interrogated soil metagenomic libraries for the presence of a biosynthetic gene, AHBA synthase, common to the ansamycin family of BGCs. They identified a group of BGCs with close similarity to rifamycin but with the addition of tailoring genes that would be predicted to generate a novel structure. One BGC was expressed in a native strain and was found to produce novel rifamycin congeners displaying inhibition of Gram-positive bacteria, a novel mechanism of action (MOA) and without rifamycin cross-resistance.

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The predicted inclusion of two or more positively charged residues*in silico* was used as a tool to conduct a chemocentric screen for likely producers of cationic peptides in Bacilli and Actinomyctes. Metabolic analysis of fermentation broths of selected *Bacillus* sp. revealed the production of two novel molecules, brevicidine and laterocidine, which displayed antibacterial activities against a range of Gram-negative pathogens, including colistin-resistant *Escherichia coli*.

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The linkage of self-resistance genes with BGCs can be used to predict novel antibiotic-encoding BGCs. These authors developed a bioinformatic tool to automate the identification of such gene/BGC pairs in sequenced bacterial genomes. This paper demonstrates the utility of this tool by predicting the presence of previously identified self-resistance genes and how this tool can be extended to identify novel BGCs.

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Through the transcriptomic analysis of four*Salinispora* sp. coupled with metabolomic analysis, the majority of BGCs were found not to be transcriptionally silent and growth-phase dependent expression was not widely observed. Key differences between similar BGCs in different strains were described, specifically in regulatory elements that can convert a BGC from silent to active.

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In an effort to improve heterologous expression, the authors deleted 15 BGCs from the talented chassis strain*Streptomyces albus* J1074. Importantly, the strain was then used to express several previously cryptic BGCs from a range of *Streptomyces* and non-*Streptomyces* species. This study demonstrates the importance of continued focus on host development for the expression of novel molecules.

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A degenerate PCR approach was used to identify clones from a soil metagenomic library that contained biosynthetic genes that are hallmarks of the calcium-dependent antibiotic class. The authors focused on the malacidin-type BGCs, from which a novel CDA was identified through heterologous expression. The malacidins are structurally distinct members of this class and exhibit antibacterial activity against Gram-positive species including MRSA and VRE. Furthermore, a novel lipid II-binding mechanism of activity was demonstrated, without vancomycin cross-resistance.

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