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NATURAL PRODUCTS - ORIGINAL PAPER

New voyages to explore the natural product galaxy

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Abstract
Natural products are a large family of diverse and complex chemical molecules that have roles in both primary and secondary metabolism, and over 210,000 natural products have been described. Secondary metabolite natural products are of high commercial and societal value with therapeutic uses as antibiotics, antifungals, antitumor and antiparasitic products and in agriculture as products for crop protection and animal health. There is a resurgence of activity in exploring natural products for a wide range of applications, due to not only increasing antibiotic resistance, but the advent of next-generation genome sequencing and new technologies to interrogate and investigate natural product biosynthesis. Genome mining has revealed a previously undiscovered richness of biosynthetic potential in novel biosynthetic gene clusters for natural products. Complementing these computational processes are new experimental platforms that are being developed and deployed to access new natural products.

Keywords Biosynthetic gene clusters · Genome mining · Integrated platform · Natural products · Secondary metabolites · *Streptomyces*

Introduction

Natural products are a large family of diverse chemical compounds that play important roles in cellular metabolism and in modulating biological systems and their host environments [7, 18, 41] including intra- and intercellular communication [6, 30], host defense [11, 25], nutrient acquisition and pathogenicity [21]. The Dictionary of Natural Products database contains more than 210,000 compounds spanning tremendous chemical diversity [35]. Those molecules classified as secondary metabolites, owing to their non-requirement for growth under standard laboratory conditions, include isoprenoids, polyketides, non-ribosomal peptides and glycosides, among others.

Secondary metabolites are incredibly important molecules for mankind with uses as antibiotics, antifungals, antitumor and antiparasitic products, and in agriculture as products for crop protection and animal health. Natural products, their derivatives and natural product-inspired molecules represent a significant proportion of existing drugs and as of 2013, 1453 new chemical entities had been approved by the U.S. Food and Drug Administration of which approx. 40% are natural products or were inspired by natural products [31].

Following the discovery of penicillin in 1928, the golden era for new antibiotic discovery began in the 1940s, when microbiologist Selman Waksman at Rutgers University exploited the ability of bacteria to produce their own antibiotics by systematically testing soil microbes, predominately the streptomycetes. This led to the discovery in 1943 of streptomycin, the first antibiotic used to treat tuberculosis, and the awarding of the Nobel Prize to Waksman in 1952. The "Golden Age" of discovery followed where the main classes of antibiotics were discovered over a period of 25 years [17]. A rapid decline in antibiotic discovery followed as pharmaceutical companies turned their attention to synthetic combinatorial chemical libraries and away from exploring Nature's chemical diversity.

However, today we are seeing a resurgence of activity in exploring natural products for a wide range of applications, due to not only increasing antibiotic resistance, but the advent of next-generation genome sequencing and new technologies to investigate natural product biosynthesis. The

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awarding of the Nobel Prize in 2015 to William Campbell and Satoshi Ōmura for the discovery of the avermectins, and to Youyou Tu for the discovery of artemisinin, is truly inspiration to continue the search for novel and impactful natural products [36].

Traditional approaches for natural product discovery have centered on microbial fermentation and screening culture extracts for desired activity. Whilst proving to be highly valuable in the golden age of discovery, these methods have generated limited value in more recent years as known molecules were found time and time again. Newer approaches that have been developed and deployed using mutagenesis with chemical mutagens or transposon mutagenesis, or manipulation of transcription through targeted engineering approaches, or ribosome engineering have resulted in some discoveries and certainly significant increases in production levels of target molecules. An alternative approach has been to identify small molecule elicitors of natural product biosynthesis such as etoposide and ivermectin that induced production of novel surugamide compounds in *Streptomyces albus* J1074 [42]. However, these approaches are somewhat limited in the scale of knowledge and discovery and there is a growing need for high-throughput and scalable approaches to dramatically expand the natural product discovery landscape. The advent and application of next-generation sequencing, coupled with new genome mining tools and more sophisticated experimental approaches, is opening up new paths to novel secondary metabolite discovery and the potential for accelerating the identification of novel bioactive natural products.

Expanding the universe of natural products: genome mining

Systematic discovery and characterization of novel secondary metabolites is therefore an essential step towards better understanding of diverse ecosystems and exploiting their chemical diversity for new applications in health, food, agriculture, and the environment [19, 39]. Genes responsible for the production of secondary metabolites are often clustered in their genomes to form biosynthetic gene clusters (BGCs) which often contain all genes required for biosynthesis of precursors, assembly of the compound scaffold, tailoring of the scaffold and additional genes for resistance, export and regulation. The first sequences of Actinomycete genomes revealed a previously undiscovered richness of biosynthetic potential that was not apparent from previous analyses of culture broths. Indeed, the number of BGCs reported was 3–10 × that of the number of secondary metabolites found in broth extracts [3, 16, 28].

The advent of next-generation sequencing technologies has revolutionized genome science by enabling scientists to explore biological diversity at an unprecedented scale, cost and quality. Application of short-read technologies such as the new Illumina NovaSeq platform has enabled massive scale sequencing but which still result in approximately 40 contigs per genome for microbial genomes (unpublished results). Fragmented genomes are far more difficult to assemble and annotate, and large, repetitive PKS and NRPS open reading frames can be particularly problematic to resolve. The advent of long-read single molecule real time technology from Pacific Biosciences has enabled almost single contig genomes to be generated which leads to better assemblies, especially of BGCs encoding modular enzymes such as type I polyketide synthases, which in turn lead to better predictions of BGCs. An informative review on next-generation sequencing of Actinobacteria offers some enlightening perspectives and recommendations [10].

To mine this wealth of sequence data and to identify secondary metabolite BGCs, advanced computational tools have been developed. Here, we highlight some of the key tools that have been developed to identify and explore novel BGCs. The comprehensive open-source BGC mining platform antiSMASH was first released in 2011 and has been updated with the current version being 4.2 [4, 5]. It identifies BGCs in genomes based on a query sequence against a built-in library of profile hidden Markov models of signature proteins experimentally characterized to be involved in the biosynthesis of particular specialized metabolites. It covers a wide range of different types of BGCs and can allow for computation of the number of BGCs and total coding capacity for BGCs of a genome from a particular microbe. ClusterFinder is incorporated as an option into the antiSMASH software which detects BGCs based on calculating the probability of a stretch of Pfam domains to constitute a BGC above a certain threshold and does not rely on signature enzymes. The outputs from antiSMASH are connected to further resources including the Joint Genome Institute's (JGI) Integrated Microbial Genomes Atlas of Biosynthetic Gene Clusters (IMG–ABC) system [12, 13].

AntiSMASH is now complemented by RODEO (Rapid ORF Description and Evaluation Online), developed by Doug Mitchell's group, that uses hidden Markov model analysis, heuristic scoring and machine learning to evaluate local genomic context of BGCs and precursors to predict putative ribosomally synthesized and post-translationally modified peptide (RiPP) BGCs and putative structures of their products [40]. RODEO was successfully deployed to identify 1315 lasso peptides within 1419 BGCs, of which 6 were further explored for their structure and bioactivity.

Sean Brady developed the Environmental Surveyor of Natural Product Diversity (eSNaPD) to identify BGCs from metagenome sequences in which sequence tags are generated by PCR from (meta)genomic DNA using degenerate primers that target conserved biosynthetic domains and can

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be barcoded to differentiate DNA from different samples in a pooled next-generation sequencing run [33]. Raw amplicon sequencing data are processed using the eSNaPD tool, which automatically cleans the input data, classifies BGCs present in the samples by performing a phylogenetic comparison to a reference set of characterized, known molecule BGCs and visualizes the results in a number of ways that aid in identifying the most desirable samples for library generation. The newly generated metagenomic library is arrayed to facilitate the identification and recovery of target clones, sequenced using position-specific barcoded primers, and eSNaPD analysis is performed to generate a detailed biosynthetic profile of the library that facilitates the identification of high-value target clones for recovery and heterologous expression.

The Prediction Informatics for Secondary Metabolomes (PRISM) structure prediction algorithm was developed by Nathan Magarvey's group at McMaster University for chemical structure prediction of up to 22 distinct natural product cluster types [37, 38]. Following open reading frame detection in a microbial genome sequence, protein sequences are analyzed and clustered using a library of hidden Markov models for secondary metabolite biosynthesis genes of known experimentally characterized gene clusters and enzymes. Identified biosynthetic information is subsequently leveraged for combinatorial prediction of secondary metabolite chemical structures. In the latest version, natural product scaffolds are modeled as chemical graphs which permit manipulation of the predicted structure at the level of atoms or bonds that can then be tagged as potential sites of tailoring reactions before combinatorialization.

As genome sequencing increases, such does the need for standardization of experimental and contextual data for BGCs to enable massive comparative and functional analyses. The Minimal Information about a Biosynthetic Gene cluster or MIBiG specification is an extension of the Genomic Standards Consortium's MIxS framework and provides a comprehensive and standardized specification of BGC annotations and gene cluster-associated metadata [8]. An MIBiG-compliant seed dataset of 1815 clusters has been constructed through a community annotation of BGCs that have been experimentally characterized. MIBiG covers general parameters that are applicable to each and every gene cluster, as well as compound type-specific parameters that apply only to specific classes of pathways.

At the JGI, we have developed IMG–ABC which we foresee as becoming the largest publicly available database of experimentally verified and predicted BGCs [13]. In the latest version of IMG–ABC, new functionality has been incorporated [12]. The biosynthetic cluster (BC) cart serves to collect the BGCs of interest for further in-depth analysis and it contains the features specifically designed to facilitate visualization and analysis of BGCs through comparing architectures of selected BGCs and the content of flanking genomic areas. The functional heatmap tool can be used to find Pfam content and BGC similarities which can be visualized through the Similarity Network tab. The ClusterScout tool uses an algorithm based on Pfam hooks, their distance and those that must be present in the cluster to identify BGCs across all publicly available isolate bacterial genomes in IMG.

Several years ago, JGI launched the Genomic Encyclopedia of Bacteria and Archaea (GEBA) project to expand the availability of reference genomes to catalog bacterial and archaeal diversity, identify novel protein families and provide better reference tools for metagenome analysis. We recently published the first 1000 genomes from this study [27]. Using the combination of antiSMASH and ClusterFinder we predicted the BGCs of these genomes and reported that species of Actinobacteria had > 100 BGCs assigned through this method, well in excess of what had been previously reported. Further analysis by us and Baltz showed that this was an inflated count related to the genomes being highly fragmented due to the short-read sequencing technology employed and the subsequent misleading use and poor predictability by ClusterFinder for the short reads and BGC assignment [1, 2]. Baltz has conducted further calculations of the number of gene clusters for a wide range of microbes using antiSMASH without ClusterFinder [1]. He demonstrated that the most gifted microbes, mostly members of the Actinobacteria, have up to 52 BGCs which can encompass up to a quarter of the total genome size. His more recent analyses have further highlighted the overestimation of validated BGCs by ClusterFinder; and the poorer correlation with genome size and uniform genome location of putative BGCs predicted by ClusterFinder [2]. Baltz also analyzed a set of uncultured microbes with relatively small genomes and showed that they encode a very limited number of secondary metabolite BGCs and proposed that uncultured microbes with small genomes are not a deep reservoir of novel natural products.

As a large-scale sequencing and genome science user facility, the JGI is expanding novel natural product diversity through its microbial, fungal, plant and metagenome sequencing programs conducted with the scientific community. For example, we recently published a comparative genomic analysis of secondary metabolite BGSs from six *Aspergillus* species and used the SMURF tool to predict secondary metabolite BGCs [20]. We conducted a comparative analysis of secondary metabolite BGCs predicted in the closely related *A. novofumigatus* and *A. fumigatus* to increase understanding of how pathogenicity and virulence is manifested. *A. novofumigatus* was found to contain 65% more clusters than *A. fumigatus,* and many of the same allergens and virulence factors suggesting that this organism is also pathogenic. We also identified putative BGCs clusters for aflatoxin, chlorflavonin, and ochrindol in

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A. ochraceoroseus, *A. campestris*, and *A. steynii*, respectively, and novofumigatonin, ent-cycloechinulin, and epi-aszonalenins in *A. novofumigatus*. We also identified new secondary metabolite BGCs in species not previously known to contain them.

The ability to link genome information and metabolite identification remains a challenge. The JGI has developed the Metabolite, Annotation, and Gene Integration (MAGI) tool that addresses these limitations by generating metabolite–gene associations via biochemical reactions based on a score between probable metabolite identifications and probable gene annotations [9]. This is calculated by a Bayesian-like method and emphasizes consensus between metabolites and genes. The probability of a metabolite identity increases if there is genetic evidence to support that metabolite, and the probability of a gene function increases if there is metabolomic evidence for that function. Inputs to MAGI are typically two data types: a metabolite identification file of LCMS features and a protein or gene sequence FASTA file. MAGI was applied to sequence data and metabolomics data collected from *Streptomyces coelicolor* A3(2) and was able to associate genes with reactions that can be ranked in *S. coelicolor* and coupling metabolomics and genomics data by scoring consensus between the two increases the quality of both metabolite identifications and gene annotations. Moreover, MAGI was found to make correct biochemical predictions for poorly annotated genes that were readily validated by the literature searches.

Expanding the universe of natural products: new experimental platforms

The advent of next-generation sequencing technologies and predictive computational tools has accelerated the identification of BGCs, yet progress in experimentally validating and characterizing BGCs and their products has been comparatively slow. New experimental technologies and innovative applications are driving new approaches to natural product discovery.

The expression of BGCs is subject to tight regulation of gene expression, post-translational processes such as protein folding, post-translational modifications, and cellular processes such as the availability of substrates and co-factors and the ability of a strain to tolerate intermediates and products. These factors limit the ability to detect and identify secondary metabolites under standard laboratory conditions and in the best cases only 10–20% of BGCs can be functionally expressed to synthesize their products.

Two approaches have successfully overcome these limitations: unlocking suppression of BGC expression in native hosts or refactoring the regulatory systems to bypass control of expression. Overexpressing positive regulators and/or deleting negative regulators have been used to activate transcription. Such a strategy was applied to discovery of novel chattamycins and new ansamycins through overexpression of activator genes [23, 44]. Alternatively, introducing strong promoters upstream of the target BGC has led to successful BGC expression and production of the corresponding molecules. Elegant work by Huimin Zhao's group at the University of Illinois used CRISPR-Cas9 technology to knock in promoters to activate secondary metabolite BGCs [34, 43]. Knock in of the *kasO** promoter upstream of the *indC-like* indigoidine synthase gene resulted in production of indigoidine in *S. albus*. Similarly, his group also showed production of the red undecylprodigiosin and blue actinorhodin in *S. lividans*. The Zhao group further showed successful production of novel NPs in multiple species including this polycyclic tetramate macrolactam.

Whilst these approaches have been demonstrated to be effective for tackling functional activation of a small set of BGCs, methods that allow for large-scale functional expression and identification of novel natural products are desired. The JGI is developing technologies that enable genomic integration and expression of large numbers of BGCs and parallel testing in a diverse portfolio of bacterial strains. So far, this technology has been deployed to over 40 species of Proteobacteria and Actinobacteria (unpublished results). Cell-free transcription-translation systems also offer a potential approach for rapid prototyping of BGCs and *Streptomyces*-based systems are under development [22, 26].

In addition to these new synthetic biology-based methods, new microbiological approaches are being employed to successfully activate BGCs and identify new natural products. Elegant work by Kim Lewis and his collaborators developed a novel way of simultaneously isolating and growing uncultured bacteria using the iChip in which a sample of soil is diluted so that a single bacterial cell is delivered to a given channel, after which the device is covered with two semi-permeable membranes and placed back in the soil [24]. Diffusion of nutrients and growth factors through the chambers enables growth of uncultured bacteria in their natural environment. A substantial number of the uncultured isolates were then able to grow in vitro. Extracts from 10,000 of these were screened for their antimicrobial activity on plates overlaid with *Staphylococcus aureus*. One such extract from a novel bacterium *Eleftheria terrae* was shown to contain teixobactin, an unusual depsipeptide which was shown to be active against vancomycin-resistant *S. aureus*.

An alternative approach is to use co-cultivation of two or more microbes to induce expression of natural product BGCs. A new study by Roberto Kolter and Jon Clardy's groups describes a transwell assay in which a complex nine strain actinomycete community was grown on solid media coupled to a liquid phase for providing nutrients and for sampling [32]. With this approach, their groups were able to identify

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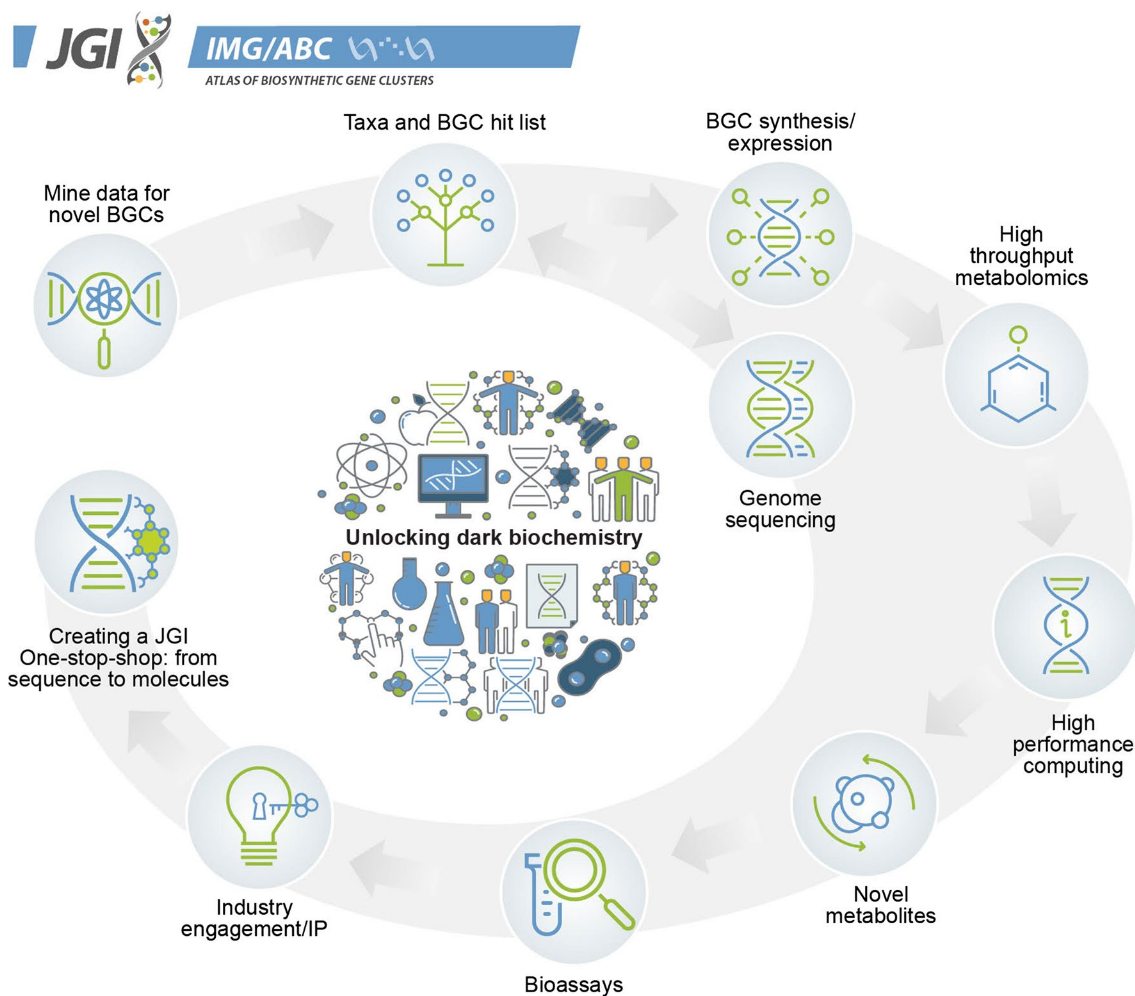

Fig. 1 Integrated natural product platform that combines genomics, functional BGC expression, metabolomics, computing and assays

amycomicin, produced by *Amycolatopsis* sp. AA4, as a potent and specific inhibitor of *S. aureus* fatty acid biosynthesis. Additionally, Hiroyasu Onaka's group discovered that mycolic acid-containing Actinobacteria induce natural product biosynthesis in *Streptomyces* sp. through direct interaction between live cells of two species [14, 15, 29]. This methodology has been deployed to identify a number of natural products that are otherwise not produced, including alchivemycin A. At the JGI we have been conducting a series of co-cultivation studies and sampling for both RNAseq and LC–MS/MS analysis. Using this integrated approach, we have so far identified 34 metabolites which we are currently characterizing and aligning to their originating BGCs.

Integrated natural product platform

With the advent of these new tools and technologies, a very real opportunity exists to accelerate the identification and characterization of novel natural products with relevant biological activity through the development of an integrated natural product platform. In this platform, capabilities in DNA sequencing, transcriptomics, computational identification and prediction, BGC expression and natural product development will be brought together in a high-throughput and scalable manner to enable the

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large-scale identification of novel natural products (Fig. 1). The JGI is currently piloting such a platform.

Conclusions

Natural products are an incredible resource of useful bioactive molecules and there still remains significant untapped diversity. The advent of new tools is enabling increased access through complete high-quality genomes, powerful genome mining tools and new experimental platforms to produce and characterize the resulting molecules. Bringing these technologies together into integrated platforms that can address large-scale natural product characterization projects will enable further exploration of the natural product galaxy.

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