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Omics-based natural product discovery and the lexicon of genome mining

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Genome sequencing and the application of omic techniques are driving many important advances in the field of microbial natural products research. Despite these gains, there remain aspects of the natural product discovery pipeline where our knowledge remains poor. These include the extent to which biosynthetic gene clusters are transcriptionally active in native microbes, the temporal dynamics of transcription, translation, and natural product assembly, as well as the relationships between small molecule production and detection. Here we touch on a number of these concepts in the context of continuing efforts to unlock the natural product potential revealed in genome sequence data and discuss nomenclatural issues that warrant consideration as the field moves forward.

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Introduction

Natural products (NPs) and their derivatives comprise about half of the clinically approved medicines including many of our most important antibiotics [1–3]. Despite the considerable gains made from studying this resource, industry's investment in NP research waned in recent decades. This paradigm shift can be attributed to a number of issues including the continued rediscovery of known compounds and the alternative use of combinatorial libraries as a discovery platform [4]. Enthusiasm for NPs research was renewed however following the revelation that only a small fraction of the biosynthetic potential observed in microbial genome sequences could be linked to the small molecules whose biosynthesis they encode [5]. This observation, coupled with the development of new omic-based technologies, has re-invigorated

the field and spawned the concept of genome mining as an alternative method for NP discovery [6].

Natural product genome mining takes a bioinformatics first approach to small molecule discovery. It provides opportunities to specifically target biosynthetic gene clusters (BGCs) that can be predicted to encode interesting new compounds or new derivatives within structural classes of interest. Genome mining has benefited from a better understanding of the biosynthetic logic of natural products [7,8] and enhanced computational approaches that automate the process by which BGCs are detected and characterized [9,10^{••}]. These advances led to the development of the widely used web tool antiSMASH [5], while others such as PRISM (Prediction Informatics for Secondary Metabolome) [11] and IMG/ABC [12] represent more recent additions. Access to these tools has brought NP analyses into the mainstream, making it possible for anyone with a genome sequence to make an informed bioinformatic assessment of specialized metabolism in their organism of choice. Other recent tools such as plantiSMASH [13] and fungiSMASH [14] improve the analysis of BGCs in plants and fungi, respectively. The MIBiG repository of experimentally characterized BGCs (<http://mibig.secondarymetabolites.org/index.html>) represents another important milestone and provides a straightforward platform to perform comparative BGC analysis and metabolite prediction.

While there remains considerable excitement about the discovery opportunities afforded by genome mining, our ability to identify BGCs from sequence data continues to far exceed our ability to identify the NPs they encode. This bottleneck results from the fact that only certain types of BGCs, such as NRPSs and modular, type I PKSs [15], are generally amenable to bioinformatic linkage with their products. Many others require considerably more investment, usually in the form of a genetic knock-out or heterologous expression. Both of these experimental approaches have unique challenges that vary with the organism being studied. Nonetheless, progress has been made in the automation of heterologous expression systems [16] and in retro-biosynthetic approaches to link orphan BGCs to known compounds [17] thus suggesting that this bottleneck may ultimately be overcome through advances in synthetic and computational biology. Meanwhile, the relatively few BGCs that have been experimentally characterized (1393 BGCs in MIBiG as of May 2017) relative to the considerably larger number of microbial NPs discovered over the last 50-plus years (52,395

microbial NPs [18]) raises the generally understated possibility that much of the apparent genetic potential observed in bacterial genome sequences has already been realized.

The many innovative approaches that have been applied to NP genome mining [9,19,20], including the activation of silent BGCs [21,22*,23,24], have been extensively reviewed in recent years. Here we highlight a number of gaps that remain in our basic understanding of the relationships between BGCs and the NPs whose biosynthesis they encode. We also discuss a number of nomenclatural issues relevant to genome mining, which at present lacks a unified lexicon.

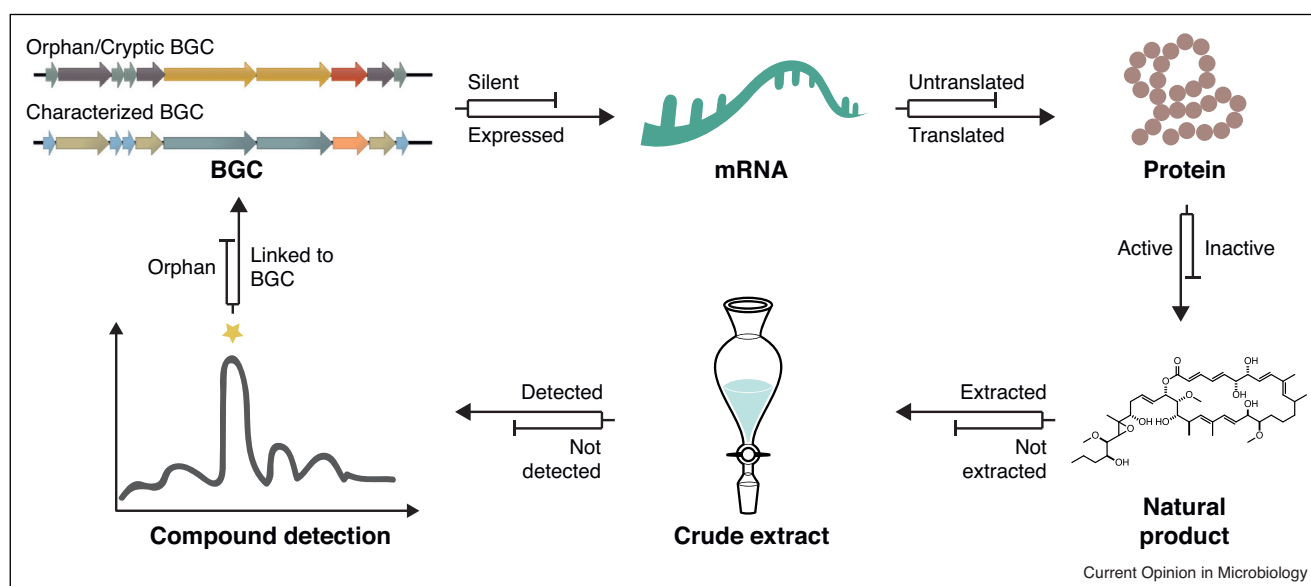
The lexicon of genome mining

The large number of BGCs observed in genome sequence data has generated considerable interest in developing new techniques to find their products. This has also led to the use of a variety of terms to describe those BGCs that cannot readily be linked to their products. Over the last decade, these unassigned BGCs have been called 'silent', 'cryptic', or 'orphan', all of which have been applied with the same intended meaning. While the term orphan was proposed 10 years ago by Harold Gross [25], the term cryptic has also come into common usage [24]. An NCBI PubMed search of the terms orphan and cryptic biosynthetic gene clusters

suggests that the former is only slightly favored. Given the appropriate context, (i.e., a BGC) the implications of orphan or cryptic seem readily apparent. While there is clear precedent for the use of both terms to describe BGCs that have yet to be linked to the small molecules they encode, we have followed the original suggestion by Gross for the use of orphan [25]. Complicating the matter is the alternative use of the term orphan to describe compounds whose cognate BGC has not been identified [26]. This later usage appears fitting given the more colloquial interpretation of orphan as a lack of parents or affiliation. Thus, an orphan compound can be considered one that lacks affiliation with its parent BGC (Figure 1).

As mentioned, 'silent' has also been commonly used to describe orphan BGCs. However, the term silent also carries the connotation of transcriptional inactivity. Surprisingly, there's very little data to support the frequently cited suggestion that orphan BGCs are transcriptionally silent. Yet transcriptional silencing is only one of many possible reasons why so many BGC remain orphan. Alternative explanations can be found at the levels of translation, functional protein assembly, and small molecule extraction and detection (Figure 1). Furthermore, the products of many orphan BGCs are readily detectable yet there is simply no practical method to link them to the appropriate gene cluster. This failure to connect the two

Figure 1



The lexicon of omics-based natural product discovery. Biosynthetic gene clusters (BGCs) can be orphan or cryptic, that is, they have not been linked to the small molecules they encode. They can be transcribed into mRNA (expressed) at levels sufficient to yield detectable compound, or if transcription falls below these levels, the BGC is considered to be silent. Assuming mRNA is translated into protein, NP assembly will depend on the availability of precursors and co-factors required for protein activity. Once produced, many NPs are likely missed because they are either not extracted or not detected with the methods employed. The vast majority of microbial NPs that have been discovered over the last 50-plus years have yet to be linked to their respective BGC and thus the compounds remain orphan.

data points leaves both compound and BGC orphaned. BGCs have also been termed silent when their encoded NPs are known but not detected in the native organism. In this case, the implied silence could be due to a failure at any step in the gene to small molecule pipeline. Thus, as with the term orphan, the use of silent is most meaningful when appropriately qualified. For example, it is clear that a transcriptionally silent BGC is one that is expressed below the levels where the cognate small molecules can be detected. It is much less clear how to interpret a BGC labelled as silent in the absence of transcriptome, proteome, or metabolome data. Here we reserve the term silent to describe BGCs for which there is evidence of ineffectual transcriptional activity.

Transcriptomics

To what extent are orphan BGCs expressed? Surprisingly, this remains largely unknown despite extensive efforts being devoted to their activation [24,27]. Global expression analyses have rarely been applied to distinguish between silent and expressed BGCs in a given organism. In cases where this has been addressed, methods such as PCR probing of cDNA have been used [28]. Microarrays have also been used to assess BGC expression. In the case of *Streptomyces coelicolor*, this approach revealed that eight of 22 BGCs were expressed at higher levels following the transition from exponential to stationary phase [29,30**]. Microarrays also revealed that 12 of 18 BGCs were expressed in wild-type *Myxococcus xanthus*, thus suggesting that many orphan BGCs may not be transcriptionally silent [31]. More recently, next-generation sequencing was employed in the global transcriptome analysis of *S. coelicolor* [32]. This study provided important new insight into the relationships between transcription and translation, something that remains poorly understood in the context of NP biosynthesis. Despite these advances, it can be argued that we still have a generally poor understanding of how many BGCs are transcriptionally silent under any given set of cultivation conditions.

More commonly, expression analyses have been used in association with searching for the products of BGCs following either the genetic manipulation of the wild-type strain or the introduction of the BGC into a heterologous host. Recent efforts using the former technique include micro-array based comparative transcription profiling, which led to the discovery of a series of polyketides encoded by a BGC that was silent in the wild-type strain but expressed in a $\Delta bldM$ mutant [33]. Expression analyses have also been used to demonstrate that silent BGCs can be activated in a heterologous host following genetic reconstruction [34]. While synthetic biology holds great promise for NP discovery [35], it is not always clear if an orphan BGC was transcriptionally silent in the native organism prior to genetic manipulation. This is unfortunate as evidence of compound production in the wild-type helps establish the relationships between orphan

and transcriptionally silent BGCs and can be used to guide future BGC activation efforts.

While transcriptome analyses can provide important distinctions between silent and expressed BGCs, the levels of gene expression that correlate with NP detection also remain largely unknown. These levels will undoubtedly vary with each BGC depending upon numerous factors including the structure of the NP and the detection methods employed. Furthermore, translation efficiencies can vary and thus expression alone is unlikely to be an adequate predictor of protein or NP production. For example, a recent study in *S. coelicolor* revealed that the transcription of several BGCs increased throughout growth yet their translation efficiency decreased at certain times [32]. It is also interesting to consider how the abundance of various tRNAs can affect translation efficiencies [36] and how a mutation in *bldA*, which is predicted to encode a misfolded Leu-tRNA molecule, can have a profound effect on BGC expression [37*].

Proteomics

As with transcription, we know little about genome level BGC translation. Early reports describe the detection of proteins associated with six orphan BGCs in *M. xanthus* [38]. More recently, the Kelleher group pioneered the application of proteomics in NP discovery by capitalizing on the properties and size of non-ribosomal peptide synthetases and polyketide synthases (often >200 kDa) and their unique marker ions derived from phosphopantetheinyl cofactors [39,40]. The PrISM (Proteomic Investigation of Secondary Metabolism) platform (not to be confused with the more recent PRISM genome mining tool [11]) was developed to exploit this concept and represents a 'protein-first' strategy that is complementary to bioassay-guided and genomic approaches [41]. The more recent 'Genome-Enabled' PrISM (GE-PrISM) incorporates draft genome sequences in the analysis and expands on the types of biosynthetic proteins that can be detected in a process termed 'proteogenomics' [42]. Despite the successful applications of proteome-based genome mining, DNA-based approaches remain more widely accessible and employed.

Translation also requires another level of scrutiny as some key biosynthetic enzymes require post-translational modification for activity. PKSs and NRPSs are prime examples, in that they are modified by phosphopantetheinyl transferases (PPTases), which post-translationally install a 4'-phosphopantetheine arm on the respective acyl or peptidyl carrier proteins [43]. These prosthetic groups are derived from coenzyme A and are required to convert inactive *apo*-synthases to active *holo*-synthases. This concept was recently exploited by overexpressing PPTases in actinomycetes, which led to an increased production of metabolites in 70% of the strains studied [44**]. To the best of our knowledge, this is the first example in which

the regulation of protein activity was used to elicit NP production.

Metabolomics

Improved analytical techniques have made it possible to more effectively assess the complex NP mixtures present in most crude extracts. While NMR has been used for metabolomic analyses, mass spectrometry remains the instrument of choice [45]. Driving these advances are approaches such as Global Natural Product Social Molecular Networking (GNPS), which allows for the visualization of complex metabolomic datasets based on MS/MS fragmentation patterns [46]. Other new tools such as DEREPLICATOR allow users to rapidly identify novel peptidic NPs in their datasets [47]. While metabolomic analyses have historically been performed independently of any knowledge of the associated BGCs, it has become increasingly possible to integrate these datasets to generate bioinformatic links between NPs and their associated BGCs. Comparing BGC distributions among strains with metabolomic profiles has been termed ‘pattern-based genome mining’ and used to compare large numbers of closely related bacteria [48,49]. Similar approaches were used to identify the rimosamide, detoxin, and tambromycin NPs and their associated BGCs [26,50*], with the later approach termed metabologenomics. Genomics and metabolomics have also been coupled to discover new NPs from cyanobacteria [51]. Other metabolomic approaches include peptidogenomics [52] and glycogenomics [53], where MS/MS fragmentation patterns are used to establish bioinformatic links between NPs and their associated BGCs based on adenylation domain amino acid specificity or glycosyltransferase substrate specificity, respectively. These informal links can be valuable in terms of prioritizing NPs and BGCs for study, yet do not eliminate the bottleneck that remains with experimental validation.

Considerable advances have been made in the activation of silent BGCs [22*,24,54,55] using techniques such as co-cultivation [56] or the addition of small molecule inducers [57,58]. While these methods have proven useful, it is also possible that many BGCs are active yet their small molecule products are simply missed due to the extraction methods or analytical techniques employed (Figure 1). For example, recent metabolomics analyses have shown that extraction solvents had a major impact on the metabolites detected [59,60]. As we learn more about the relationships between orphan BGCs and their products, it is likely that much of the apparent biosynthetic potential observed in microbial genomes will ultimately be linked to compounds that are in fact produced but simply missed or ignored because they do not possess the properties that allow them to be detected or make them attractive targets for discovery.

Conclusions and future perspectives

The extant number of NPs that await discovery is unknown and will undoubtedly remain so. However, a large reservoir of unexplored chemical space can be predicted [18], thus supporting the concept that NP research will continue to yield important new discoveries. One clear concept to emerge from the post-genomic era is that a majority of BGCs detected in genome sequences are orphan, that is, they have yet to be linked to the NPs whose biosynthesis they encode. We also know that the number of microbial NPs discovered to date far exceeds the number of BGCs that have been experimentally characterized. Thus, the jury remains out on how many orphan BGCs will yield structurally unique NPs versus known compounds or new derivatives thereof. This knowledge gap will be filled as the bottleneck in BGC characterization is resolved. A seldom discussed unknown is the rate at which Nature creates new chemical diversity. While this rate may not meet our demands for new drug pipelines, a major goal of synthetic biology is to speed up these processes through genetic engineering. While there have been successes in this regard [61], we undoubtedly still have a great deal to learn from the microbes themselves, which are the true masters of NP biosynthesis.

A surprising unknown remains the distinction between transcriptionally silent and expressed BGCs in wild-type strains. Without global transcriptome data, it is difficult to distinguish between silent BGCs and those that are expressed yet fail to be connected to their small molecule products. Thus, it is possible that some discovery efforts may be better focused at the post-transcriptional level. Additional unknowns relate to the temporal dynamics of NP biosynthesis. For example, it is unclear what level of gene expression correlates with protein or NP detection. Also the time frame between gene expression and NP assembly is poorly understood, as are the half-lives of active biosynthetic enzymes. Finally, expression levels may not mirror protein assembly due to differences in translational efficiencies or post-translational regulation. Advances in all of these areas will provide important insight for future NP discovery efforts.

Despite these significant unknowns, there are numerous examples where orphan BGCs have yielded new compounds [34,40,62–64], thus supporting the concept that genome mining and associated omic approaches hold considerable promise for NP discovery. The continued development and application of these technologies will help fill the knowledge gaps discussed above and allow for the better exploitation of NP resources. As the field moves forward, having a unified lexicon to describe BGCs and the NPs whose biosynthesis they encode provides the best mechanism to effectively communicate among NP researchers and with the scientific community at large.

Conflict of interest

The authors declare no conflict of interest.

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