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Mining genomes to illuminate the specialized chemistry of life

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Abstract

The common language that unites all life is carried out by small-molecule chemical signals. These specialized metabolites have evolved to impart diverse cellular and ecological functions, and are broadly applied in medicine, agriculture and nutrition. The rapid accumulation of genomic information has revealed that the metabolic capacity of virtually all organisms is vastly underappreciated. Initially mainly in bacteria and fungi, genome mining technologies were pioneered to accelerate metabolite discovery. Recent efforts are now being expanded to all life forms, including plants, animals and protists, and new integrative omics technologies allow increasingly effective mining of this diversity.

Introduction

Genomically encoded small-molecule chemicals are the common language that unites all life, from single cells to communities of organisms. Whereas many biochemicals are shared among large swaths of the tree of life, some molecules are biosynthesized only by a select subset of organisms and/or are specific to certain ecological niches. These specialized metabolites, also called natural products or secondary metabolites (see glossary, as well as refs.[1–3] for in-depth discussions of the definitions of these terms), range in size, shape and complexity, from small terpenes and phosphonates to large and heavily posttranslationally modified gene-encoded peptides. As such, they have often evolved to impart diverse cellular, intraspecies and interspecies functions that perform key roles in physiology and in simple to complex ecosystems. Specialized metabolites provide organisms, from single-cell microorganisms to multicellular plants and animals, with some of their most distinguishing chemical features of color, smell, taste or toxicity. In other words, the blend of specialized metabolites endowed to an organism makes it unique.

Most specialized metabolites have been identified through experimental discovery approaches that take advantage of a chemical or biological feature of the expressed molecule to guide its isolation. Molecules such as penicillin, estradiol and caffeine are just a small selection of nature’s chemical bounty that has had profound societal impact (Figure 1a).

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Strikingly, the rapid accumulation of genomic and transcriptomic information in recent years has revealed that the metabolic capacity of virtually all organisms is vastly underappreciated, with millions of additional molecules awaiting discovery[4–6].

Genome mining seeks to harness gene-based big data methods to expedite the concomitant discovery of specialized metabolites and their biosynthetic genes. With increasing technological improvements in genome sequencing, early mining experiments of relatively simple microbial genomes have been followed in recent years by much more complex genomes and metagenomes of plants, animals and other eukaryotic organisms that organize their biosynthesis genes differently (Figure 1). Additionally, to truly arrive at a deeper understanding of life's chemistry, genome mining approaches are being developed that provide insight into the functions that these molecules perform in physiology and ecology. Here, we address the why, what, where and how of genome mining and discuss key challenges in figuring out what nature is verbalizing.

Why we mine and what to mine

Historically, specialized metabolites have been isolated and characterized from biological samples collected from the environment or from laboratory-grown organisms whereupon organic extracts of tissues or cells are chemically and biologically analysed. In this way, natural chemicals have been identified, dating back to the year 1803 with the isolation of morphine from opium poppy[7]. While analytical chemistry tools continue to improve in sensitivity and speed to aid the discovery process[8], trends over the past decades have shown a clear deceleration of the discovery of novel structure chemotypes versus the rediscovery of well-known molecular families with subtle chemical modifications[9]. Genome mining has the potential to change the discovery rate and makes it possible to identify molecules that would otherwise remain under the radar. This is exemplified by the fact that after the publication of the genome sequence of the model organism *Streptomyces coelicolor* A3(2)[10], which had been heavily studied for about half a century already and for which around a dozen (types of) specialized metabolites had been known, genome mining has since led to the discovery of seven additional ones from diverse classes: the nonribosomal peptides coelibactin[10] and coelichelin[11], the sesquiterpene (+)-epi-isozizaene[12], 2-alkyl-4-hydroxymethylfuran-3-carboxylic acids[13], the SCO-2138 RiPP[14], the polyketide coelimycin P1[15] and a new set of partially characterised arsenopolyketides[16]. As such, genome mining has key differences and advantages as compared to the use of analytical techniques alone. First, it can access specialized metabolites that may not be produced under the growth conditions studied. Second, the approach inherently connects any discovered molecules to their biosynthetic genes, allowing for heterologous expression and bulk production. This is particularly significant because many medicinally valuable molecules are isolated from dwindling natural resources or organisms that are difficult to cultivate, and genome sequencing typically requires much less biomass than the quantities that are required for structural elucidation.

The motivations for genome mining have largely tracked those of the natural products community at large: historically, this has primarily been the exploration of life's biochemical prowess, the understanding of physiology, and the pursuit of therapeutics. In the last century, the first specialized metabolites were linked to their biosynthetic genes, usually from cloned DNA fragments that could be used to complement mutations in these genes[17–19]. In the 2000s, genome sequencing started to mature, and the biosynthetic logic of some major classes of medicinal natural products, including polyketides, nonribosomal peptides and terpenoids, had been deciphered to some extent. The newly sequenced genomes often harbored homologs of genes encoding the biosynthesis of these classes of compounds, but which had not been associated with a metabolic product. Heterologous expression of these ‘orphan’ biosynthetic genes resulted in the discovery of several novel natural products, including triterpenes from the *Arabidopsis* genome[20] and the hybrid peptide-polyketide aspyridones from the genome of the model filamentous fungus *Aspergillus flavus*[21]. Since these proofs of concept, countless new members of established major compound classes have been discovered through genome mining.

Genome mining is also contributing to the ongoing fundamental search for chemical and biosynthetic novelty in nature. Several specialized metabolites harboring chemical moieties unprecedented for their class, such as furanone[22,23] and benzo[a]tetraphene[24] polyketides, and aminovinylcysteine based ribosomally processed peptides[25], were discovered through genome mining. Even among known specialized metabolites, there are numerous structures for which the biosynthetic machinery was only recently elucidated — often through genome mining — such as for the piperazate[26], thiotetronate[27], oxazolone[28,29], isoxazole[30], indolyloxazole[31], alkyne[32,33], N-nitroso[34], and diazo[35] moieties, polybrominated phenolics from marine bacteria[36], plant-like isoquinoline alkaloids in diverse fungi[37] and vinca alkaloids from medicinal plants[38]. As new biosynthetic reactions and structural classes are discovered, our ability to reliably predict orphan genes for novel molecular scaffolds will continue to improve. Still, there are many biochemical scaffolds for which the genetic basis is still completely or mostly a mystery, such as the polycyclic ethers found in dinoflagellates[39], or the ladderanes produced by anammox bacteria[40,41]. There are doubtlessly numerous novel scaffolds not represented among known specialized metabolites that will one day be discovered through genome mining.

Our understanding of ribosomally-synthesized peptides has particularly benefited from the rise of genome mining, thanks to the fact that their structures can often be relatively easily predicted from genomic data. One class of these peptides, the RiPPs (for ribosomally-synthesized and post-translationally modified peptides)[42], is particularly noteworthy for its broad distribution across all three domains of life and our growing knowledge of its diversity of peptidic modifications[43]. Not to be confused with non-ribosomal peptide synthetase products, new structural families of RiPPs continue to be discovered such as the spliceotides[44] and epipeptides[45] from bacteria, dikaritins[46,47] from fungi, and the lyciumins[48] from plants. Ribosomally-derived specialized metabolites are not always RiPPs and can range remarkably in size, from small molecules like the pyrroloquinoline alkaloid ammosamide[49,50] to small proteins like three-finger toxins from spitting cobras[51] and venom proteins from spiders[52,53]. Similar discovery trends can also be seen in the other major biosynthetic lineages, where the mining of genomes has resulted in a growth of chemical and biochemical knowledge.

What else is there to mine and what happens to genome mining after we have exhaustively identified all specialized metabolite scaffolds? Based on the inventory of known specialized metabolites and those that are already connected to biosynthetic genes, the future remains bright considering the efficiency and breadth of new strategies for genome mining and given the increased extent of resources available for mining. Even in cases where the mining of orphan genes leads to re-discovery of previously reported specialized metabolites, solace comes in the discovery of new enzymes and biosynthetic knowledge that may have biotechnological utility.

In recent years, new motivations for genome mining have emerged from two new areas of research: microbiomes and synthetic biology. In microbiome research, the mining of specialized metabolites and the genes encoding their biosynthesis provides a window into the mechanisms responsible for key phenotypes mediated by the microbiome, such as pathogen suppression[54,55] or host immunomodulation[56]. Moreover, it potentially enables the design of synthetic microbial consortia that can be used as live therapies or biologicals[57–59], based on genome-based prediction of the chemical capabilities of individual strains. In synthetic biology, pathways are being mined from genomes mainly as a source of enzymological diversity, which are started to be used as ‘parts’ for metabolic engineering of novel molecules with desirable properties[60]. This may enable combinatorialization of enzymes[61] or even computer-aided design[62] to create ‘new-to-nature’ molecules.

Where to mine

Since genome mining is predicated on the availability of omics data, growth in the field has relied on improvements in sequencing technologies. To this day, the majority of genome mining has been conducted on bacterial genomes, which, given their comparatively small size and low repeat content, dominate publicly available genomic databases (Figure 1c). Further simplifying the mining process within bacteria is their propensity to physically cluster genes in operons and biosynthetic gene clusters (BGCs, see Box 1) for cooperative biosynthesis of specialized metabolites. This has allowed researchers to readily formulate hypotheses regarding the biosynthesis of molecules of interest, even in cases where substrates and enzymes have no precedent. For instance, genes clustered with a gene known to be involved in the biosynthesis of a specialized metabolite are often promising candidates to focus experiments that aim to identify other genes involved in its biosynthetic pathway.

Soil microorganisms, and in particular the actinomycetes, were already a popular source of specialized metabolites in the pre-genomic era and were thus obvious targets for early sequencing and mining efforts. The first genomes of *Streptomyces*, *Salinispora* and *Saccharopolyspora* species pre-2008 revealed that the actinomycetes were more metabolically rich than originally thought, with many species dedicating over 10% of their genomic space to the production of dozens of specialized metabolites[10,63–65]. This trend has now been observed in many other environmental bacteria, especially those with large genomes in excess of 10 Mb. The filamentous marine cyanobacterium *Moorea producens*, for instance, devotes roughly one-fifth of its genome in this manner[66]. Due to decreasing costs of bacterial genome sequencing, recent efforts have ballooned in scale to mining 10,000–100,000+ genomes at a time for novel molecules[67,68].

The specialized chemistry of uncultivated bacteria that dominate the microbiota of animals, plants and other host organisms has also been examined through genome mining, highlighting the importance of microbial metabolites in mitigating health and disease within their hosts. Whether it be human gut bacteria[69], plant rhizosphere microbial communities[70], or marine sponge microbiota[71], the metagenomic mining of the microbial dark matter of life is quickly revealing that microorganisms are indispensable for the host's chemical fitness. In cases where there is no living host, such as in soils, seawater and even the air, environmental DNA (eDNA) further reveals the exquisite metabolic capacity of the earth's microbiota[72,73]. While attempts to exploit eDNA as a genetic resource for natural product discovery were initiated already two decades ago[74], better computational infrastructure such as reference databases[75] and profiling software[76], as well as massively increased sequencing volumes, have now turned this into a promising technology. Indeed, innovative efforts have now led to the engineered production of drug leads directly from the mining of soil eDNA samples[77,78].

Filamentous fungi, such as *Aspergillus nidulans* and *Penicillium chrysogenum*, have also long been known to cluster their genes for the biosynthesis of, for example, the antibiotic penicillin or the carcinogenic toxin aflatoxin[18,79]. While fungi and bacteria share many of the same hallmark secondary metabolic pathways, they also feature distinctive enzymatic reactions such as the reducing iterative polyketide synthases (PKSs) that produce the cholesterol-reducing agent lovastatin[80]. With their larger genomes, fungi also encode many more BGCs than the most prolific bacteria. The fungus *Aspergillus tanneri* NIH1004 has 95 BGCs[81], setting it up as the most fruitful amongst the fungi.

Long thought to be a uniquely microbial phenomenon, it is now becoming increasingly clear that BGCs are found throughout the tree of life (Box 1). Land plants dwarf all other organisms for known specialized metabolites (Figure 1d). Plant molecules, like the anticancer drug taxol, the plant hormone gibberellin or caffeine (which functions as an insecticide yet is best known as a constituent of coffee and other caffeinated drinks), dominate the literature on specialized metabolism with over 145,000 described molecules. Early experiments connecting plant chemistry and genes relied upon sequencing expressed sequence tag libraries and transcriptomes. In recent years, plant genomics has gained traction, revealing the genomic context of specialized metabolism. The triterpene thalianol in *Arabidopsis* was one of the first plant compounds for which it was shown that its encoding genes are chromosomally clustered[82], yet in a manner very much unlike the bacterial BGCs. Genes within plant BGCs are typically not organized in tight operons but rather with large intergenic regions that can span up to a few hundred kb in stretches, and as such, genes are typically transcribed separately[83]. Recent plant omic studies have connected genes to the production of iconic opioid, cannabinoid, and vinca alkaloid plant molecules, leading to renewable fermentation opportunities for their robust production[38,84,85].

The success of the plant community in connecting genes to specialized chemistry has opened the floodgates to other eukaryotic systems that each harbor distinctive chemistry. For instance, some of the most notorious environmental toxins are produced by diverse marine microalgae. Recently, a BGC was established in the diatom *Pseudo-nitschia multiseries* for the global production of the amnesic shellfish toxin domoic acid[86]. By contrast, dinoflagellates produce arguably the largest and most complex chemicals known from nature, polyether toxins such as brevetoxin and maitotoxin[87]. While biosynthesis genes have yet to be identified for these dinoflagellate compounds — perhaps due to their massive genomes that regularly exceed humans and assemble into liquid crystalline chromosomes[88] — the recent assembly of the toxic ~6.4-Gb *Amphidinium gibbosum* draft genome revealed an abundance of suspected PKS and nonribosomal peptide synthetase (NRPS) genes[89]. On top of this, the recent reconstruction of hundreds of genomes of plankton species from metagenomic data provides a rich set of unexplored genomic data to mine for specialized metabolic diversity[90].

Historically, the anthropocentric bias of biomedical research has led scientists to qualify metabolites isolated from many animals as distinct from bacterial, fungal and plant specialized metabolites. However, a more impartial perspective should recognize that many animal specialized molecules are chemically related to and perform functions similar to their non-animal counterparts. While in some cases, animal-derived specialized metabolites are biosynthesized by specialized microbiome members[91,92], the biosynthetic capacities of the animal itself should not be underestimated. Humans, for instance, produce numerous steroid hormones such as estradiol, cortisol and aldosterone, the thyroid hormone triiodothyronine, and even the antiviral ribonucleotide 3′-deoxy-3′,4′-didehydro-CTP[93]. The recently discovered routes from bird[94,95] and mollusc[96,97] genomes to produce complex polyketides as well as a novel sesquiterpene biosynthetic pathway from flea beetles[98] exemplify the chemical ingenuity of animals themselves in making important molecules key to their fitness and survival. In some cases, such pathways have been horizontally acquired from bacteria, as is evident for the β-lactam antibiotic biosynthetic genes found in the genome of the springtail *Folsomia candida*[99,100], but in most documented cases mentioned above, their biosynthesis seems to have evolved independently, indicating that considerable quantities of distinct chemistry may be discovered though mining animal genomes.

Now that eukaryotic genome sequencing is becoming more routine, we anticipate that genome mining projects will soon extend to all organisms (Box 2). While there have been sporadic reports of specialized biosynthetic genes and gene clusters being functionally elucidated from, for example, the nematode *Caenorhabditis elegans*[101], the fruit fly *Drosophila melanogaster*[102] and the seaweed *Digenea simplex*[103], large swaths of organisms such as arthropods, cnidarians and other invertebrates are understudied for their biosynthetic capacities yet well-known for their specialized chemistry.

How to mine — identifying and prioritizing candidates

A range of computational approaches has been developed to automatically identify the sets of genes that encode specialized metabolic pathways across genome sequences (Figure 2). Many of these approaches have originally been developed for bacteria (and sometimes for fungi and plants), but the principles employed have the potential to be extended to other life forms. Below, we review these methodologies and the taxa they support, and what would be required to extend them into new taxonomic spaces.

The physical clustering of enzyme-coding genes in BGCs greatly facilitates the identification of biosynthetic pathways. While BGCs are highly variable in terms of gene content and often strain-specific due to their rapid evolution and frequent horizontal gene transfer[104], they often do possess common properties in the form of enzyme families that are responsible for the catalysis of biochemical reactions central to the biosynthesis of entire specialized metabolite compound classes. This feature has made it possible to largely automate the identification of BGCs in genomes. Widely used software tools such as antiSMASH[105] and PRISM[106] employ profile Hidden Markov Models (pHMMs[107]) of protein domains to identify gene combinations encoding enzyme families that are signatures for specific pathway types. While both these tools generally provide very similar results, development of antiSMASH has focused more on functional and comparative analyses, while PRISM has specialized in combinatorial predictions of chemical structures that can be used for automated matching with mass-spectral data. The use of pHMMs is very reliable for identifying BGCs encoding many well-established types of biosynthetic machinery such as PKSs, NRPSs and known classes of RiPPs, but risks overlooking less studied and wholly novel classes of BGCs. Probabilistic BGC prediction methods such as ClusterFinder[108] (which is also integrated into antiSMASH) and DeepBGC[109], or comparative genomics approaches that identify metabolism-associated nonsyntenic blocks of genes between genomes are more likely to detect non-standard BGCs, but have higher false-positive rates. In addition, for RiPPs, specialized tools have emerged for the identification of BGCs encoding the production of distant members of known classes or members of altogether novel classes. Some of these, like BAGEL[110], use pHMM-based detection techniques similar to those seen in antiSMASH and PRISM. Others either make use of bait-based approaches (using specific query enzymes to identify loci that contain homologues of it)[111,112] or use machine-learning approaches to identify potential precursor-peptide-encoding genes, the hits of which can be prioritized using either metabolomics-based matching[113] or comparative genomics to identify operons that are taxon-specific and are therefore deemed to encode a specialized metabolic function[114]. For publicly available genomes, BGCs identified using antiSMASH can be interactively browsed in online databases such as IMG-ABC[115] and antiSMASH-DB[116]. Recently, it has become clear that in plants, specialized metabolic pathways are sometimes encoded by BGCs[83] (Box 1), and specific algorithms have been devised for their detection[117,118]. However, there are also many examples of pathways in plants that are encoded by sets of genes distributed across multiple chromosomes instead of being located in a single gene cluster. When extending genome mining approaches to unexplored parts of the tree of life, it remains to be seen to what extent genes in these taxa will be clustered. Some recent evidence suggests that the phenomenon of gene clustering also occurs in protists; for example, the domoic acid biosynthetic pathway in the diatom *Pseudo-nitzschia multiseries* was shown to be encoded by a four-gene gene cluster[86]. However, gene cluster detection algorithms originally devised for bacteria may require considerable optimization to make them effective for studying protist or animal genomes. Efforts to adapt antiSMASH for detecting BGCs in plants in a new tool called ‘plantiSMASH’[118] showed that, for this to be effective, new libraries of pHMMs focused on plant enzymology needed to be constructed, and the algorithm had to be adjusted to account for the considerably larger (and more variable) intergenic regions found in plant genomes[106].

Computational predictions often lead to an overabundance of candidate specialized metabolic pathways that could be investigated, necessitating prioritization in some way. Given that the chemical structures of hundreds of thousands of specialized metabolites have been elucidated, a considerable number of these will be responsible for the biosynthesis of known molecules or their closely related variants. Hence, dereplication is required to assess whether molecules and biosynthetic genes are novel compared to those ones that have been discovered and characterized earlier. The simplest way of doing this is based on sequence information: if a BGC of interest is highly similar in sequence to a gene cluster that has been experimentally linked to a known specialized metabolite, it likely codes for the production of the same molecule. In 2015, a community effort established the Minimum Information about a Biosynthetic Gene cluster (MIBiG)[75], a data standard and online repository for depositing annotations and metadata on BGCs for which a product has been identified. The antiSMASH pipeline for BGC identification automatically compares each identified BGC against this repository of ~2,000 BGC of known function. When studying large numbers of genomes at once, BGC sequence similarity networks[108] can be utilized to identify ‘gene cluster families’ (GCFs) that cluster together with MIBiG reference clusters. The BiG-SCAPE software framework automates the process of generating these networks and facilitates their interactive exploration, which makes it possible to quickly explore the biosynthetic diversity within hundreds or even thousands of prokaryotic genomes at once[119]. It remains to be seen to which extent this technology is universally applicable across the tree of life. For example, it was recently shown that plant triterpene biosynthetic loci may be highly similar in terms of domain composition, while having evolved independently and leading to divergent chemical outcomes[120]. These analyses suggest that at least certain categories of biosynthetic pathways in plants through combinatorilization of a limited set of enzyme families, of which the members can have different catalytic activities or regioselectivities. Hence, for pathway types and organisms in which gene evolution is largely decoupled from gene cluster evolution, more automated phylogenetic methods need to be developed to perform comparative analysis at the gene level as well as the gene cluster level. Beyond plants, it should not be excluded that this is the case for other eukaryotic branches of the tree of life as well.

Identification, dereplication and prioritization workflows can be further improved by combining the information from the genome sequence with data obtained from analytical techniques[121]. For instance, if the same or similar molecules are produced by different organisms, they can be expected to harbor the same or similar biosynthetic genes. Pattern-based genome mining[122] (also known as metabologenomic correlation analysis[123,124], Figure 3a) correlates patterns of spectral data (most commonly liquid chromatography (LC) mass spectrometry (MS) features) to the presence of homologous biosynthetic genes across strains. This approach (reviewed in detail here[125]) has mostly been pioneered in bacteria, for which sufficiently large numbers of genomes and metabolomes can be obtained. In a recent metabologenomic correlation study, gene cluster families (GCFs) were linked to a MS network, leading to the discovery of the tyrobetaine metabolites[126]. Recently, the mathematics behind the association scoring was improved and formalized in a software tool called NPLinker[127]. The advantage of this technology is that no prior knowledge on biosynthetic mechanisms is required to link molecules to gene clusters, as it is purely based on correlations. A strategy that establishes genomic–metabolomic co-occurrence patterns has great potential to mine the genomes of poorly studied organisms, even when virtually nothing is known about a taxon's enzymology.

Another approach that also harnesses analytical chemistry to improve genome mining predictions is the correlation of mass shifts in tandem MS fragmentation patterns to a BGC's bioinformatically predicted building blocks (Figure 3b). At first, semi-manual approaches were developed that allowed matching of peptides (peptidogenomics[14]) and glycosylated specialized metabolites (glycogenomics[128]) to BGCs. More recently, this matching has been automated for peptides in algorithms like Pep2Path[129], RiPPquest[130] and MetaMiner[131]. The latter algorithms, which focus on RiPPs, could also be very relevant for finding novel peptidic metabolites in uncharted taxa, as recent evidence is emerging that RiPPs are produced not only by bacteria, but also by fungi[132], plants[48] and animals[133]. Going forward, the bigger challenge will be to extend these approaches beyond peptides to specialized metabolites in general[125].

Instead of partial structural information from mass spectra, fully elucidated chemical structures can also be used to identify new biosynthetic pathways and aid in dereplication. There are many specialized metabolites for which the chemical structure is known but the biosynthetic genes are not. For drug discovery purposes, this may pose a major problem, given the considerable effort wasted elucidating the chemical structure of a known molecule. Recently, an innovative method called GRAPE/GARLIC was established[134] to tackle the puzzle that is 'connecting genes to molecules' for polyketides and nonribosomal peptides in an automated fashion: by breaking down known specialized metabolite structures into their biochemical building blocks and retro-biosynthetically matching these with building blocks predicted to be incorporated into molecules based on BGC sequence information, the authors were able to identify thousands of putative matches between gene clusters and molecules. Of around 16,831 BGCs, around 2,500 had best matching scores to reference molecules that were so low that they are very likely to encode the biosynthesis of novel products. While this may seem relatively little, one should consider that the remaining set of ~14,000 BGCs is enriched with many near-copies of BGCs from highly studied taxa for which large numbers of genomes have been sequenced. The retro-biosynthetic principle, while useful, seems largely limited to bacterial polyketides and nonribosomal peptides, and expanding retro-biosynthetic algorithms to other life forms will require considerable expansions of our knowledge of their biosynthetic routes. Training more generic models for enzymatic mechanisms based on large-scale experimental data are needed here, as well as high-throughput assays on 'enzymatic dark matter' from unexplored taxa to provide the required training data for such models.

The presence of specialized metabolites can also be correlated to biosynthetic genes' transcriptional levels in different conditions or across different tissues (Figure 3c). For example, the biosynthetic pathway for ingenol mebutate from *Euphorbia* plants was unraveled by identifying members of relevant enzyme families that were highly expressed in seeds[135]. Similarly, another recent study analysed the production of the defense metabolite falcarindiol by tomato across seven different biotic stress treatments, to identify relevant enzyme-coding genes that were upregulated in conditions when increased amounts of the molecule were observed[136]. This principle seems universally applicable and is widely useful for accelerating genome mining efforts.

Indeed, in plants, coexpression analysis has already been frequently used with success to identify genes that show similar expression patterns across a large number of samples, within the same species or even cross-species[137]. Often, this is done by using one or more ‘bait’ genes, which are predicted or even known to belong to a pathway of interest, to recruit additional members of that pathway[138,139]. However, unsupervised approaches are also being developed, which can be used to predict candidate pathways without prior knowledge. These methods rely on detecting coexpressed modules of genes given a set of transcriptomic samples, a procedure for which a range of algorithms is available[140]. Recently, the identification of coexpression modules was shown to effectively and comprehensively retrieve genes implicated in methionine-derived aliphatic glucosinolate biosynthesis in *Arabidopsis thaliana* and *Brassica rapa*[141]. A key factor in the success of this study was the use of a graph clustering method that allows modules to overlap in their gene content, which makes sense given the fact that plant specialized metabolic enzymes are often promiscuous and may have dual functions in multiple pathways. In general, the advantage of coexpression approaches seems to be that they are generally applicable, also when the genes encoding a pathway of interest are only partially clustered or not clustered at all. Moreover, for eukaryotes with complex genomes that are hard to assemble contiguously, coexpression-based approaches could also be performed on the basis of fragmented genome assemblies or transcriptome assemblies. A challenge for these approaches is how to find the right combination of conditions that distinguishes expression patterns of a pathway of interest most effectively from those of other pathways, without requiring massive amounts of expensive transcriptome sequencing. One possible strategy to do this would be to first generate (targeted or untargeted) metabolome data for a variety of samples, before choosing which samples are prioritized for RNA sequencing. Alternatively, integrative approaches could be developed that leverage structural information from metabolome data (for example, mass shifts and predicted substructures) to help prioritize which sets of coexpressed enzyme-coding genes are most likely responsible for the production of a given metabolite.

How to mine — function-first approaches

No matter how powerful modern genome mining approaches are to identify the genomic basis for chemical diversity, these methods are relatively blind and untargeted — usually, a molecule’s physiological and ecological importance is only considered at the very end, after structural characterization and elucidation of its biosynthetic pathway. Function has traditionally been investigated only in a very narrow sense, that is, by considering hits in activity assays relevant to human health and prosperity, to the neglect of physiological and more subtle ecological functions. Functions such as the arthropod-attracting capabilities of geosmin and 2-methylisoborneol terpenoids from streptomycete bacteria[142] or the conferring of heat stress resilience by flavonols by regulating levels of reactive oxygen species[143] were only identified decades after these metabolites were structurally characterized. To truly deepen our understanding of the fundamental roles of these molecules in biology and to allow for more targeted approaches to leverage them in, for example, drug discovery, it will be crucial to devise methods to help prioritize biosynthetic pathway candidates based on the specialized metabolite’s predicted function.

A good example of such a ‘function-first’ method, which has already gained traction, is based on the co-localization of genes within the same BGC that are indicative of function. For example, the colocalization of iron transport genes with biosynthetic genes led to the discovery of siderophore molecules, such as coelichelin and salinichelins in bacteria[144], and sideretin from plants[145] (and this principle has recently been generalized[146]). The colocalization of resistance genes or duplicated genes resembling antimicrobial targets within BGCs offers a more generalizable approach to the discovery of bioactive molecules with specific cellular targets (Figure 4a). This approach, called target-directed genome mining, was first validated with the rediscovery of the thiolactomycin antibiotics as fatty acid synthase inhibitors from orphan bacterial BGCs that contain an open reading frame predicted to be a resistance gene[27], associated with target modification of the FabF fatty acid ketosynthase. Newer studies colocalizing putative target-modifying resistance genes with BGCs to identify compounds with activities against the resistance gene target include the proteasome inhibitor fellutamide B from the fungus *Aspergillus nidulans*[147] and topoisomerase inhibitors pyxidicylines from the myxobacterium *Pyxidicoccus fallax* An d48[148]. A clever twist on this resistance gene-guided approach led to the discovery of the fungal sesquiterpenoid aspterric acid as a potent herbicide, by deploying the fungal dihydroxy-acid dehydratase self-resistance gene as a transgene to render plants resistant to aspterric acid[149]. In order to automate the resistance-based genome mining procedure, a web service called the “Antibiotic Resistant Target Seeker” (ARTS) was developed to identify BGCs containing likely self-resistance genes, suggesting they code for the production of specialized metabolites with specific biological targets[150]. Intuitively, the approach is probably applicable to any organisms in which biosynthetic pathways are genomically clustered, as long as there is sufficient selective pressure for the resistance genes to co-cluster (through facilitating co-expression and co-inheritance with the pathway). While resistance-based genome mining is a breakthrough as a key function-first strategy, the vast majority of BGCs do not contain self-resistance genes or other genes that unambiguously indicate a specific function. Hence, there is a great need for development of additional strategies to generate hypotheses about the function of the molecules produced by the remaining majority of pathways. We believe that, again, the essence of these approaches will be in combining genomics with other types of data. Below, we outline three possible ways in which this could be achieved.

A first possibility would entail correlating genomic information to bioactivities displayed by extracts (Figure 4b). There has already been some success in correlating bioactivities of extracts as determined by cytological profiling[151] to untargeted metabolomics of the same extracts using a technique called Compound Activity Mapping[152], facilitating the discovery of the quinocinnolinomycins, a new family of specialized metabolites that cause endoplasmic reticulum stress. The obvious next step will be to combine this with genomic and/or transcriptomic data to immediately identify the genes responsible for an activity of interest. Also, when cytological profiling does not give immediate insights into the mode of action of a molecule, it could be complemented with transcriptome analysis of the target cells during exposure. Indeed, machine learning methods have recently been devised that predict pharmacological properties of drug molecules, directly related to the mechanism of action, based on large-scale transcriptional response data[153]. In principle, this approach would be applicable to any life forms, for which extracts can be made, including plants, many protists, and invertebrates. This could also be done through genome-wide association studies that map phenotypes to genetic variation within a species, as has been successfully practiced to discover the cucurbitacin gene cluster responsible for the bitter taste in cucumber[154].

A second way to perform function-first genome mining would be to study the effects of the expression of BGCs on other community members within their native ecosystem, and, optionally, how they relate to emergent properties of such an ecosystem (Figure 4c). This applies primarily to microbial ecosystems and microbiota associated with plant or animal hosts. For example, metatranscriptome data from soil microbial communities were recently used to investigate the ecological roles of BGCs from novel bacterial clades identified through metagenomic binning; coexpression of BGCs with iron starvation response genes or antimicrobial resistance genes thus indicated roles for their products as siderophores or antimicrobials[155]. This concept could be extended by also looking at coexpression across species, that is, correlating the expression of putative antibiotic biosynthesis BGCs with stress responses in other organisms in the community to identify the likely target organisms. The expression of specific BGCs could also be correlated to microbiome-associated phenotypes[156] that a community confers to its host, such as disease suppression or stress resilience, to identify which molecules are likely to be responsible for mediating these phenotypes. In host organisms, such as plants and animals, expression of particular biosynthetic pathways can also be linked to functions by studying the effects on the microbiome composition; for example, a recent study linked specific triterpene pathways to either the promotion or inhibition of specific rhizosphere microbiome community members, which highlighted their specific roles in microbiome modulation[157].

A third strategy for function-first genome mining would be combining (sub-)structure prediction from sequence with structure-based prediction of biological activities and macromolecular targets (Figure 4d). Both of these prediction tasks are currently highly prone to error, but significant progress is being made on both fronts, so that a robust platform may become a reality in the not-too-distant future. Several tools are currently emerging that can predict the core scaffolds of key classes of specialized metabolites from sequence information with increasing accuracy and detail[105,106,158,159], and several efforts are underway to complement these with additional predictions of tailoring and cyclization reactions[106,160]. Also, genome-based structure predictions could be integrated with metabolomics-based (sub)structure predictions[161,162], which could confirm or guide routes through biochemical reaction space. Based on all these developments, considerable improvements in specialized metabolite structure prediction from genome and metabolome data can be expected in the near future. At the same time, within the field of computational drug discovery, methods are emerging that allow predicting macromolecular targets of drug molecules based on their chemical structures[163]. For example, the algorithm SPIDER dissects specialized metabolites into pharmacophore-sized fragments and predicts which proteins a compound targets by comparison to a library of 13,695 chemical structures of molecules of known function from the Collection of Bioactive Reference Analogs

(COBRA)[164]. This method successfully predicted polypharmacological features of the macrolide archazolid A. Similarly, in another recent study, a deep learning model was trained that could successfully predict antibiotic activities of molecules with only limited chemical similarity to those used for training[165]. When, in the future, both sequence-based metabolite structure prediction and structure-based macromolecular target prediction become increasingly accurate, they could be coupled together to predict biological targets directly from gene cluster sequences. The recently published PRISM4 pipeline provides a first step in this direction, using support-vector machines to predict the activities of the molecular products of gene clusters based on their predicted structures[166]. For the moment, this strategy is likely to be relevant mostly for bacteria and fungi, and to some extent for plants; however, when synthetic biology approaches[60] and *in vitro* expression systems[167] increasingly allow experimental characterization of large sets of enzymes from animals and protists, opportunities will likely emerge to apply this strategy in these taxa as well.

Altogether, the biosynthetic gene identification and prioritization is moving towards the incorporation of an increasingly large number of different data types. Moving forward, the pioneering approaches will likely harness an even larger number of data types simultaneously.

How to mine — testing candidates

Fundamentally, there are three types of approaches to identify the metabolic product(s) of a BGC: 1, heterologous expression in a model organism; 2, genetic manipulation of the native host; and 3, *in vitro* reconstitution.

Heterologous expression involves the cloning (also known as 'capture') of a BGC into a plasmid, cosmid or artificial chromosome, possible manipulation of the BGC, transfer into a genetically-tractable heterologous host, and testing for the presence of metabolic products compared to the unmodified heterologous host[168–170]. When possible, heterologous expression is a highly desired approach, because it enables both facile scale-up of metabolite production for structural elucidation and biological testing, and manipulation of the captured BGC for biosynthetic investigations and analog production. The large size of many BGCs has spurred the development of cloning methods that can capture BGCs directly from genomic DNA, such as transformation-associated recombination (TAR) in yeast[171,172], Linear-Linear Homologous Recombination (LLHR) in *E. coli*[173], or PfAgo-based artificial restriction enzymes *in vitro*[174]. One benefit of these PCR-free techniques is that it avoids mutation of the BGC, making sequence verification unnecessary. BGCs can also be cloned and assembled using PCR-based techniques, but since sequence verification of large BCGs by Sanger sequencing can be a bottleneck, doing so using next-generation sequencing technologies[175] will likely gain popularity.

Heterologous expression has some notable potential challenges: promoters and ribosome-binding sites (RBSs) may not be recognized, genes may require RNA splicing, proteins may require chaperones, post-translational modification or transport to organelles, required metabolic precursors or cofactors may not be present, or the heterologous pathway could encounter metabolic bottlenecks due to non-optimal enzyme stoichiometry. If the pathway's reactions are impeded to different extents, heterologous production could result in the production of metabolic intermediates or shunt products instead of the "true" specialized metabolite. Conventional wisdom states that employing heterologous hosts that are phylogenetically close relatives to the organism from which the BGC originates improves the chances of success, but exceptions to this dogma are known[176]. Techniques such as CRAGE[177] aim to streamline testing a BGC in a multitude of heterologous hosts, increasing the chances of at least one succeeding. Research dedicated to developing genetic toolkits for a variety of organisms will be crucial to streamline the heterologous expression of BGCs from organisms not closely related to classic model organisms.

Synthetic biology approaches aim to circumvent the aforementioned challenges associated with heterologous expression by 'refactoring' the candidate biosynthetic genes and/or engineering heterologous hosts ('chassis') optimized for heterologous expression of biosynthetic pathways. Chassis have been developed that provide metabolic precursors and post-translational modifications required for specific classes of specialized metabolism or to inactivate competing metabolic pathways. Refactoring usually entails bringing candidate biosynthetic genes under the control of well-characterized promoters and RBSs, elimination of introns and organellar targeting signals, and codon optimization[60]. However, gaps in our understanding of these cellular processes—for instance, how codon optimization affects gene expression and protein folding—still limit the rationality of our refactoring efforts. Several streamlined workflows for refactoring candidate biosynthetic genes have been described[178,179]. The use of combinatorial libraries[180] and independently tunable promoters[181] can help optimize the stoichiometry of biosynthetic genes *in vivo*. While fully synthesizing refactored BGCs *de novo* instead of refactoring captured BGCs is currently still prohibitively expensive for all but the best-funded projects, we expect this practice to become widespread as gene synthesis costs continue to decline.

Alternatively, the candidate gene(s) can be inactivated or repressed in their native host, followed by testing for the loss of, or decrease in the quantity of, a metabolite compared to the wild-type host[182]. To more thoroughly establish the gene–metabolite link, ideally a genetic complementation experiment should also be carried out[182]. The biggest drawback to this approach is that it can be difficult or impossible to manipulate genes in non-model organisms, but thankfully this situation is improving thanks to the broad host range of CRISPR–Cas9 technologies. The emergence of CRISPR–Cas9-based 'microbiome editing' technologies[183,184] has even made it possible to knock out genes in specific members of a complex microbiome.

Reconstitution of the pathway *in vitro* provides some advantages orthogonal to the *in vivo* approaches above, such as allowing for easier identification of pathway intermediates, determination of enzyme kinetics and substrate specificities, and quick optimization of the pathway enzyme stoichiometry[167]. However, *in vitro* reconstitution can be challenging if the metabolic precursor(s) or order of the enzymes in the metabolic pathway is unknown, or if any of the enzymes are insoluble, unstable or cannot be purified.

Once a metabolite has been identified as being the product of the candidate genes, its identity will need to be established. Depending on the method that was used to select the candidate genes, one may already have a hypothetical structure or chemical class. The act of 'dereplication' seeks to quickly identify whether the metabolite is, or is closely related to, any known molecules. Some currently popular approaches to dereplication are based on MS-MS spectral networking (such as GNPS[8]), MS-MS spectral-substructure matching (such as VarQuest[185], MS2LDA[162] and CSI:FingerID[161]) and NMR spectral clustering (such as SMART[186]), but it is worth remembering that dereplication tools are only as effective as the databases/training data that underlie them. If the molecule is likely novel, structural elucidation will be necessary. Nowadays, this is most commonly achieved through 2D-NMR techniques, with a slow uptick in the application of computer-assisted structure elucidation[187] (CASE) technologies. X-ray crystallography (occasionally aided by the crystalline sponge method[188]), and more recently, microcrystal electron diffraction[189], can also provide important insights into challenging structural elucidation problems.

Finally, some recent studies circumvent biological experimentation altogether by chemically synthesizing the predicted products of a BGC[190–193]. BGCs for RiPPs and non-ribosomally synthesized peptides are particularly amenable to this approach, as the structures of their products are highly predictable and their production can be streamlined through solid-phase peptide synthesis. Although doubt about the true identity of the BGC's product remains, this approach has yielded molecules with promising biological activities[190–192].

Conclusions and future perspectives

The study of the chemistry of life has been brought to a next level by genome mining technologies initially developed in microorganisms. Now that large-scale genome sequencing is expanding to all branches of the tree of life, there is a great opportunity to port and extend genome mining technologies to other life forms and engage in truly global studies of life's chemistry. At the same time, the microbial field has much to learn from scientists studying humans and mammals, who have been very effective at identifying physiological roles of mammalian specialized metabolites, whereas microbiologists have perhaps focused too much on metabolite functions restricted to inhibiting or killing other organisms. Additionally, plant biologists' extensive experience using gene expression analysis to link genes to molecules and identify their functions may become incredibly useful to the microbial field to acquire deeper perspectives into the physiological roles of many metabolites that have appeared 'inert' for so long. Finally, protists and invertebrates provide an immense uncharted biological diversity that is mostly untapped and likely to yield numerous new and surprising findings. All in all, great potential presents itself in unifying these diverse scientific communities to find common ground between molecules and genes that may have seemed unrelated for so long. This will facilitate arriving at a deeper fundamental biological understanding of the ecological and physiological roles of life's chemistry, and more effectively leveraging it for the common good in medicine, agriculture and nutrition.

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Glossary

The terms ‘secondary metabolites’, ‘natural products’ and ‘specialized metabolites’ are often used interchangeably. Below we attempt to delineate the differences in how we use these terms:

Secondary metabolite

A metabolite that is not strictly required for growth and development, as opposed to a primary metabolite; often important for the survival of an organism in its environment

Natural product

A small molecule originating from a living organism or natural source that is often prized for its medicinal properties or other biological activities of utility to humanity

Specialized metabolite

A natural compound of limited clade- or niche-specific distribution with a specialized role in ecology or physiology

Other terms:

Biosynthetic gene cluster

(BGC) A set of genes that is physically collocated on the chromosome and together encodes the production, regulation and transport of one or more specific metabolites

Gene cluster family

(GCF) A set of similar biosynthetic gene clusters across strains or species, the members of which are responsible for the production of the same or very similar metabolites

RiPP

Ribosomally synthesized and post-translationally modified peptide, biosynthesized through the action of tailoring enzymes on a ribosomally-translated precursor peptide

Polyketide synthase

(PKS) Enzyme involved in the biosynthesis of polyketide metabolite; some form modular assembly lines of multidomain proteins, while others act as stand-alone enzymes

Nonribosomal peptide synthetase

(NRPS) Enzyme involved in the polymerization of amino acids or other organic acids into peptide metabolites without involvement of the ribosome

Profile Hidden Markov model

A computational model, trained on a multiple-sequence alignment of a protein family, used to assess whether other proteins are also part of (or related to) this family

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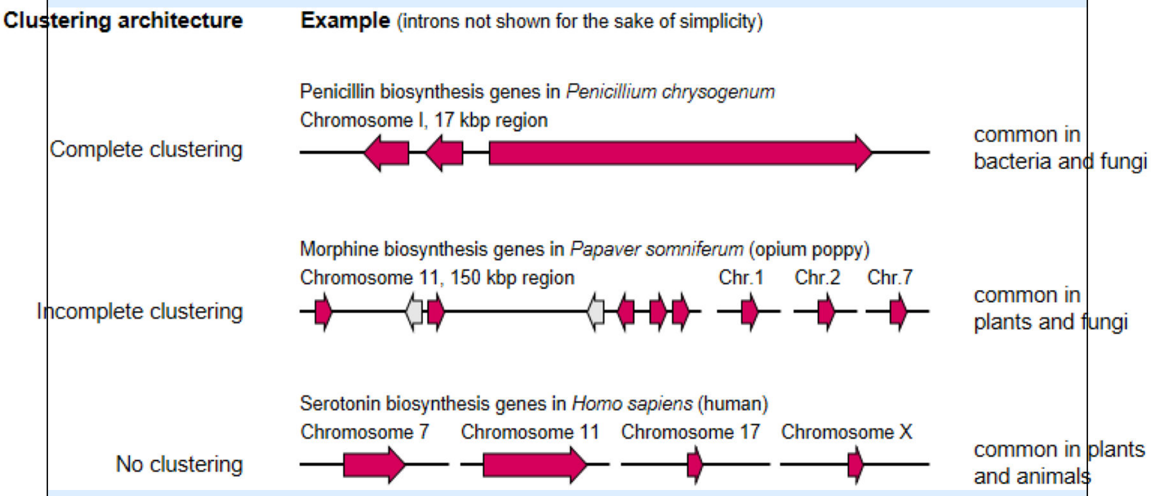
Box 1:

gene clustering in specialized metabolism


Clustering architecture
Example (introns not shown for the sake of simplicity)
Penicillin biosynthesis genes in *Penicillium chrysogenum*
Chromosome I, 17 kbp region
Complete clustering
common in bacteria and fungi
Morphine biosynthesis genes in *Papaver somniferum* (opium poppy)
Chromosome 11, 150 kbp region
Chr.1
Chr.2
Chr.7
Incomplete clustering
common in plants and fungi
Serotonin biosynthesis genes in *Homo sapiens* (human)
Chromosome 7
Chromosome 11
Chromosome 17
Chromosome X
No clustering
common in plants and animals

In most organisms, genes involved in specialized metabolic pathways are encoded contiguously on the chromosome in so-called biosynthetic gene clusters (BGCs). The extent to which biosynthetic genes are clustered differs between different taxonomic groups, and specifically between the plant, fungal and bacterial kingdoms, which show increasing degrees of gene clustering. As an illustration, in the model actinomycete bacterium *Streptomyces coelicolor*, 22 BGCs have been experimentally characterized and linked to products (including two single enzyme-coding genes), and for none of the corresponding pathways is there evidence of being encoded in multiple genomic loci. On the other hand, out of the 23 BGCs experimentally characterized in the model fungus *Aspergillus nidulans*, at least three pathways have been shown to be split over multiple loci: those for the biosynthesis of austinol / dehydroaustinol[194], emericellin[195] and nidulanin A[196]. In the model plant *Arabidopsis thaliana*, only four pathways have been experimentally shown to be encoded by BGCs: those for the biosynthesis of thalianol, marneral, arabidiol, and tirucalladienol. While several other pathways seem to show partial clustering[157,197], the pathways for the biosynthesis of glucosinolates, flavonoids, strigolactones, arabidopyrones, camalexin and 4-hydroxyindole-3-carbonyl nitrile seem to be (almost) devoid of clustering. Still, even in plants, BGCs are an attractive target for pathway discovery, as they provide ‘low-hanging fruits’ that can be straightforwardly identified in genome sequences[5]. In protists, several examples of BGCs have been reported[86,198], while in animals, not much is known about gene clustering. Yet, a recent global synteny network analysis makes clear that gene order in mammals is clearly nonrandom and may have large functional repercussions[199].

There are several hypotheses for why the genes for specialized metabolic pathways are clustered on the genome. The four main ones are the following:

1. **Coordinated gene expression**. In bacteria, given that transcription and translation occur in the same cellular location, the biophysics of transcriptional regulation favors co-regulation of operons located near

the gene encoding a pathway-specific regulator[102]. In fungi and plants, there is evidence that clustered genes are co-regulated through epigenetic modification of chromosomal regions[200,201].

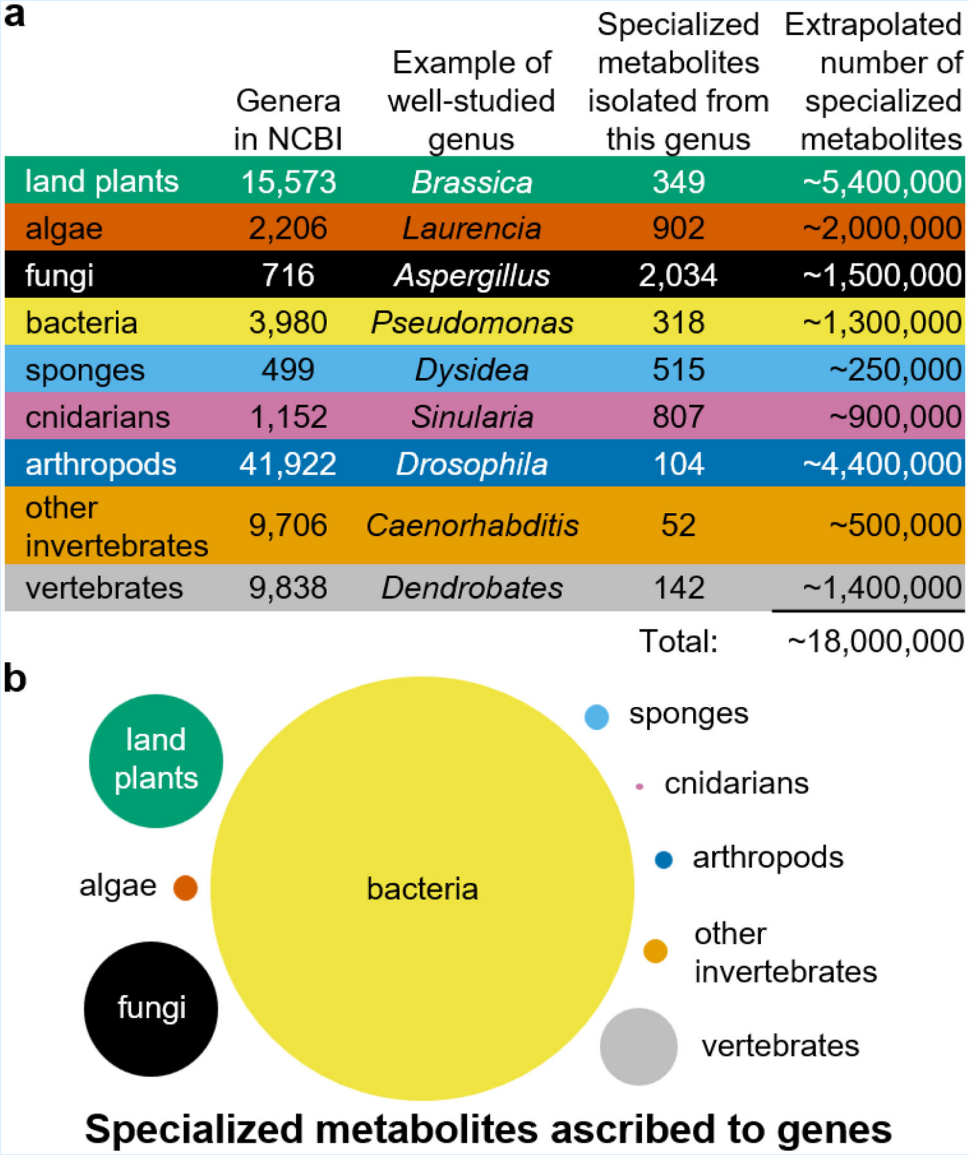
2. **The selfish operon hypothesis.** Given that horizontal gene transfer of BGCs, but also their deletion, occurs frequently in bacteria and fungi, the ‘survival’ of BGCs in the biosphere may depend on their ability to spread to other strains and species; clustering may increase chances of genes to be jointly transferred[202]. This can be supplemented by a ‘persistence hypothesis’, stating that clustered genes are less likely to be interrupted by a segmental duplication and therefore more likely to survive as a unit[203].

3. **Avoiding toxic intermediates.** According to this hypothesis, clustering of genes is an adaptation against the accumulation of toxic pathway intermediates. Clustering promotes co-inheritance of the entire pathway, so that (sub)lethal genotypes carrying only part of the pathway are avoided[204].

4. **Co-adaptation through co-inheritance.** Many clusters in plants and fungi have formed in dynamic chromosomal regions as part of evolutionary arms races with competing species[205]. Especially in sexual organisms, rapid adaptation of pathways may only be possible when co-adapted alleles of the underlying genes are not constantly separated by recombination events. This has recently been proposed to drive repeated and independent evolution of gene clusters encoding phenylpropanoid degradation pathways in fungi[206].

Box 2:

How much is there to mine?



a

	Genera in NCBI	Example of well-studied genus	Specialized metabolites isolated from this genus	Extrapolated number of specialized metabolites
land plants	15,573	*Brassica*	349	~5,400,000
algae	2,206	*Laurencia*	902	~2,000,000
fungi	716	*Aspergillus*	2,034	~1,500,000
bacteria	3,980	*Pseudomonas*	318	~1,300,000
sponges	499	*Dysidea*	515	~250,000
cnidarians	1,152	*Sinularia*	807	~900,000
arthropods	41,922	*Drosophila*	104	~4,400,000
other invertebrates	9,706	*Caenorhabditis*	52	~500,000
vertebrates	9,838	*Dendrobates*	142	~1,400,000
			Total:	~18,000,000

a: Estimating the total number of specialized metabolites by multiplying the number of specialized metabolites reported for a relatively well-studied genus — assumed to be representative — by the number of genera for the type of organism. These could be overestimates because genera may share specialized metabolites, or underestimates because more specialized metabolites may be discovered for the chosen genus or more genera may still be discovered. Number of specialized metabolites were sourced from Natural Product Atlas[207] for *Pseudomonas* and *Aspergillus*, and from Dictionary of Natural Products for all other genera. This data considers only the isolation source, not whether the specialized metabolite was produced by the host or a microbial symbiont. **b**:

Areas indicate relative numbers of specialized metabolites whose biosynthetic genes have been identified, based on estimates made by the authors.

Both the large diversity of molecules found in nature and the even larger diversity of biosynthetic genes found in genome sequences make it clear that the chemical and enzymological space available to genome mining is vast. Yet, it is difficult to gauge just how vast it is.

Focusing on possibly the most chemically diverse clade of microorganisms, the actinomycetes, Doroghazi et al. have claimed that sequencing a well-chosen set of only ~15,000 actinomycete genomes would reveal virtually all naturally occurring GCFs in this class of bacteria[124]. They based this statement on extrapolating a rarefaction curve of GCFs, in which sampling had been corrected for phylogeny within the limits of the dataset used. A subsequent study on the diversity of NRPS gene clusters, which included a larger number of genomes and used chemical structure predictions to support family assignments, indicated no signs of saturation around 15,000 genomes, however, suggesting that genome-encoded biosynthetic diversity may be larger than previously estimated, at least for this class of pathways[158]. Similarly, Schorn et al.[208] revisited estimates of biosynthetic diversity based on a study of rare marine actinomycete genomes, which suggested that rarefaction analyses may be too conservative to estimate diversity across the biosphere, as they inherently do not take into account genomes from unsampled ecological niches and taxonomic subgroups.

A rough estimate of the total number of specialized metabolites employed by life can be made based on known biodiversity (Fig. 1b) and metabolic diversity (panel a and Fig. 1d): on the order of tens of millions. Contrasting this to the number of elucidated specialized metabolites (on the order of half a million) suggests we have merely scratched the surface of the biochemical diversity present in the biosphere. Studies on bacteria and fungi support this notion, showing that regardless of the rapid accumulation of known specialized metabolites and associated risks of rediscovery, the absolute numbers of structurally novel specialized metabolites discovered over the past 20 years has remained remarkably steady, at around 150–250 per year[9,209].

While the estimates in panel a suggest there is great potential for the discovery of specialized metabolites throughout the whole tree of life, our understanding of their biosynthesis is heavily skewed towards bacteria (panel b), likely due to the greater availability of genomic data for bacteria (Fig. 1c). Even for the relatively well-studied specialized metabolism of bacteria, our understanding of culturable species dwarfs uncultured bacteria. This could be remedied by bringing more bacterial species into culture through new sampling or cultivation strategies[210,211], or by expanding metagenomic studies of diverse environments globally, and in turn mining the resulting genomics data. Nevertheless, to spur our understanding of specialized metabolism throughout the whole tree of life, it will similarly be imperative to collect thorough genomic data for a wide variety of eukaryotic organisms.

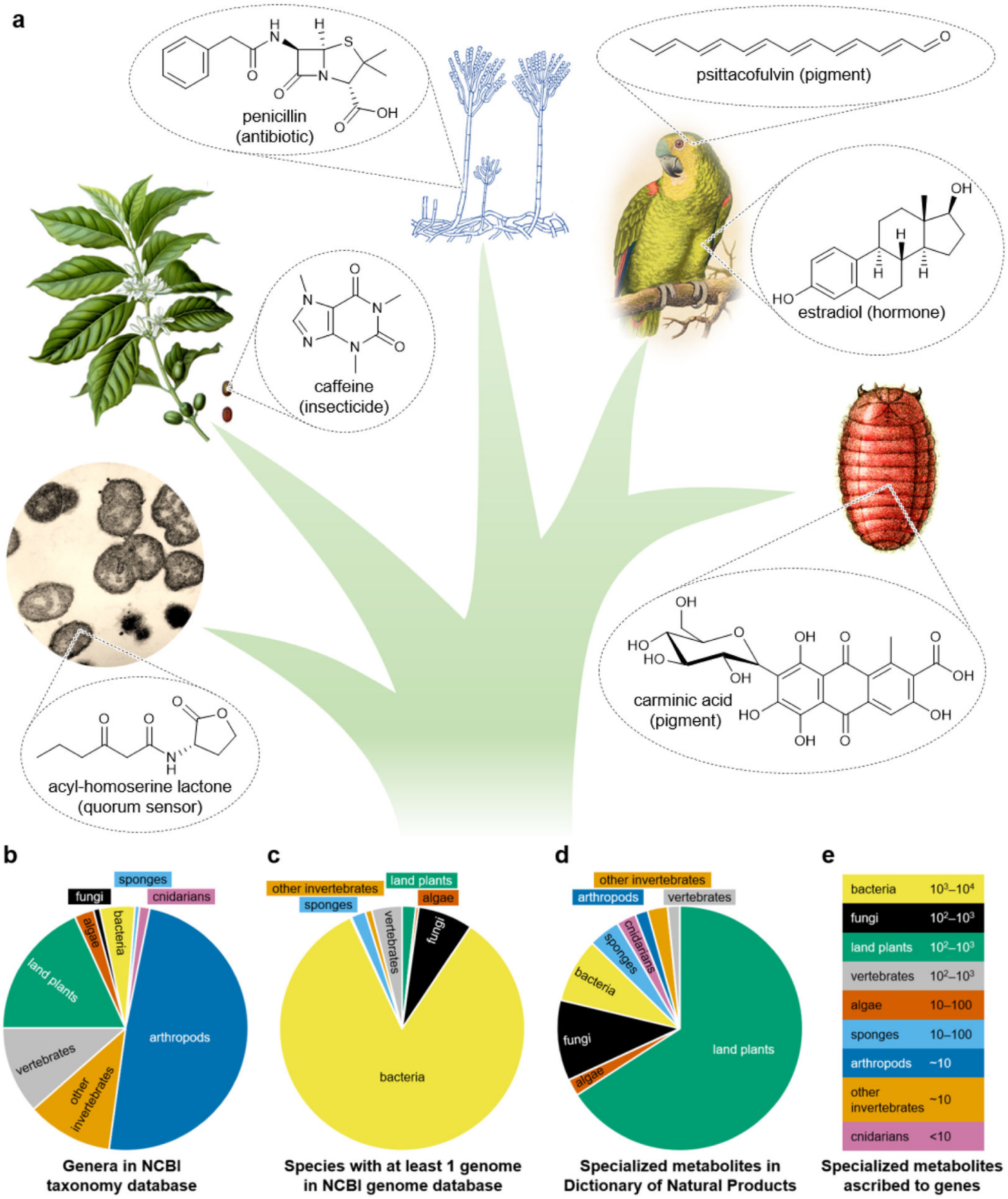
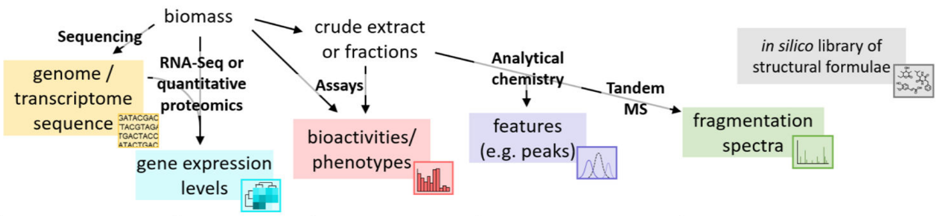


Figure 1. Life's chemical diversity.

a) Bacteria, fungi, plants and animals produce a wide range of specialized metabolites that help them thrive in their respective environments. There is a large disconnect between (b) the numbers of taxonomic genera in the biosphere (as based on the NCBI taxonomy database), (c) the numbers of genomes available for these species (based on the number of species represented in the NCBI genome database), (d) the numbers of specialized metabolites isolated (based on the number of molecules ascribed to these classes of organisms in the Dictionary of Natural Products) and, (e) the estimated numbers of specialized metabolites that have been linked to genes responsible for their biosynthesis (estimates by the authors). There is likely great potential for discovering new metabolites from animals and protists, and identifying new biosynthetic pathways from plants, animals and protists. Algae includes green, red, and brown algae, diatoms and dinoflagellates. Heterotrophic protists and archaea were not included due to the low number of specialized metabolites isolated from these organisms.


biomass
Sequencing
RNA-Seq or quantitative proteomics
genome / transcriptome sequence
gene expression levels
crude extract or fractions
Assays
bioactivities/ phenotypes
Analytical chemistry
features (e.g. peaks)
Tandem MS
fragmentation spectra
in silico library of structural formulae

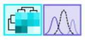
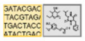
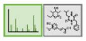
Hypothesis- generating method	Input data	Helps generate hypotheses about	Select examples	Select software pipelines
Classic genome mining		gene-chemistry relationships	• triterpenes[20] • coelichelin[11,212] Reviewed in [4,213,214]	• antiSMASH[105] • PRISM[106] • DeepBGC[109] • CO-ED[29] Focussed on RiPPs: • RODEO[111] • DecRiPPter[114]
Function-directed genome mining		gene-chemistry- function relationships	• thiolactomycin[27] • aspterric acid[149] • siderophores[146]	Target-directed genome mining: • ARTS[150] • FRIGG[215] Genome neighborhood analysis: • EFI-GNT[216]
Coexpression analysis	, optional	gene-chemistry relationships	• steroidal glycoalkaloids[137] • 4-hydroxyindole-3-carbonyl nitrile[139] reviewed in [5,140,217,218]	• WGCNA[219] • CoExpNetViz[220] • PlantiSMASH[118]
Gene-metabolite correlation analysis		gene-chemistry relationships	• Proteomining[221] • Falcarindiol[136]	
Pattern-based genome mining / Metabologenomics	or 	gene-chemistry relationships	• tambromycin[123] • tyrobetaines[126] • several *Salinispora* BGCs[122] • zealexin biosynthesis through association mapping in maize[222] • chemically guided functional profiling[223] reviewed in [125,224]	• NPLinker[127] • EFI-CGFP[216]
Gene-phenotype correlation analysis	or 	gene-function relationships	• Bitterness in cucumber[154] • Flavobacterial NRPS-PKS in disease suppression[55]	correlation pipelines designed for pattern-based genome mining may be adapted for this purpose
Activity-guided genome mining		gene-chemistry- function relationships	• lomaiviticin[225]	
Gene-metabolite substructure matching	U	gene-chemistry relationships	• peptidogenomics • glycogenomics reviewed in [125,226]	• RiPPquest[130] • Pep2Path[129] • MetaMiner[131]
Retrobiosynthetic matching		gene-chemistry relationships		• GRAPE/GARLIC[134] • rBAN[227]
Spectral dereplication		novel chemistry		• VarQuest[185], • MS2LDA[162], • CSI:FingerID[161]

Figure 2.

Overview of genome mining technologies that combine genome sequence data with gene expression levels, metabolomic data, biological activity or phenotypic data, and chemical structure data. Each combination has its own strengths and may allow generating hypotheses focused on finding an unknown biosynthetic pathway for an important known molecule, discovering new metabolites with desired biological activities, or identifying potential links between metabolites and the genes and gene clusters that likely encode their biosynthesis.

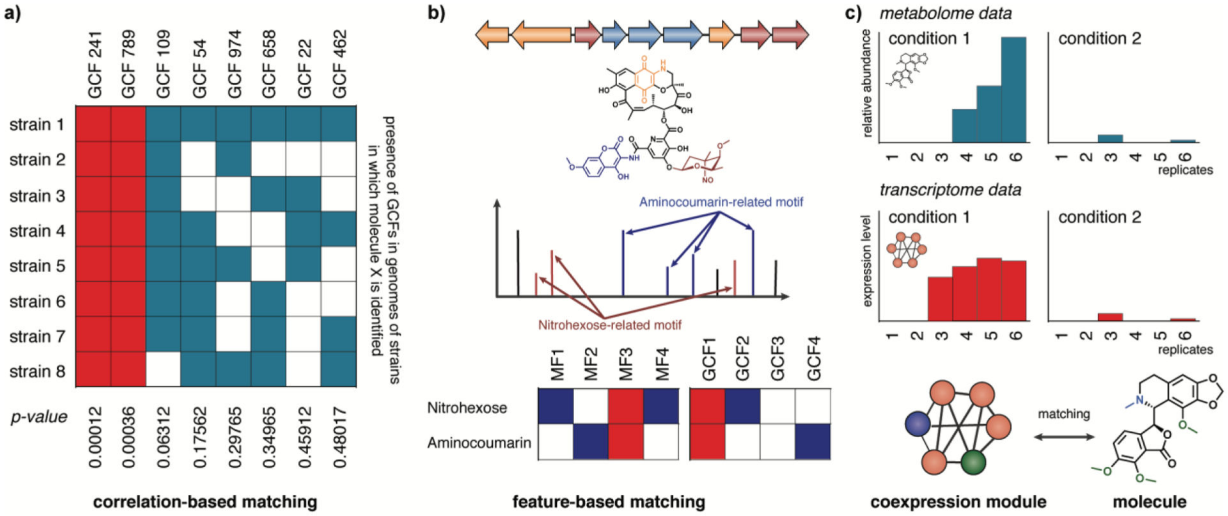


Figure 3. Linking genes to molecules using metabolomics and transcriptomics.

Several approaches have been developed to link metabolites to genes and gene clusters encoding their biosynthesis. a) In bacteria, pattern-based genome mining approaches have been developed that match families of molecules (related by spectral similarity) to gene clusters families (GCFs, related by sequence similarity) through metabologenomic correlation[123], which identifies which GCFs co-occur strongly in the same strains where a given metabolite is observed. b) Molecules can also be connected to genes and gene clusters through feature-based matching, in which chemical features (substructures and modifications that are either manually annotated or identified using algorithms that identify motifs in MS/MS data) are linked to genes and gene modules that are known to be responsible for the biosynthesis of such features. c) Transcriptomic data can also be used to identify potential biosynthetic pathways for a molecule of interest by, for example, identifying modules of coexpressed genes whose expression correlates with the presence of a given metabolite across a range of divergent conditions (for example, different biological stresses[136]).

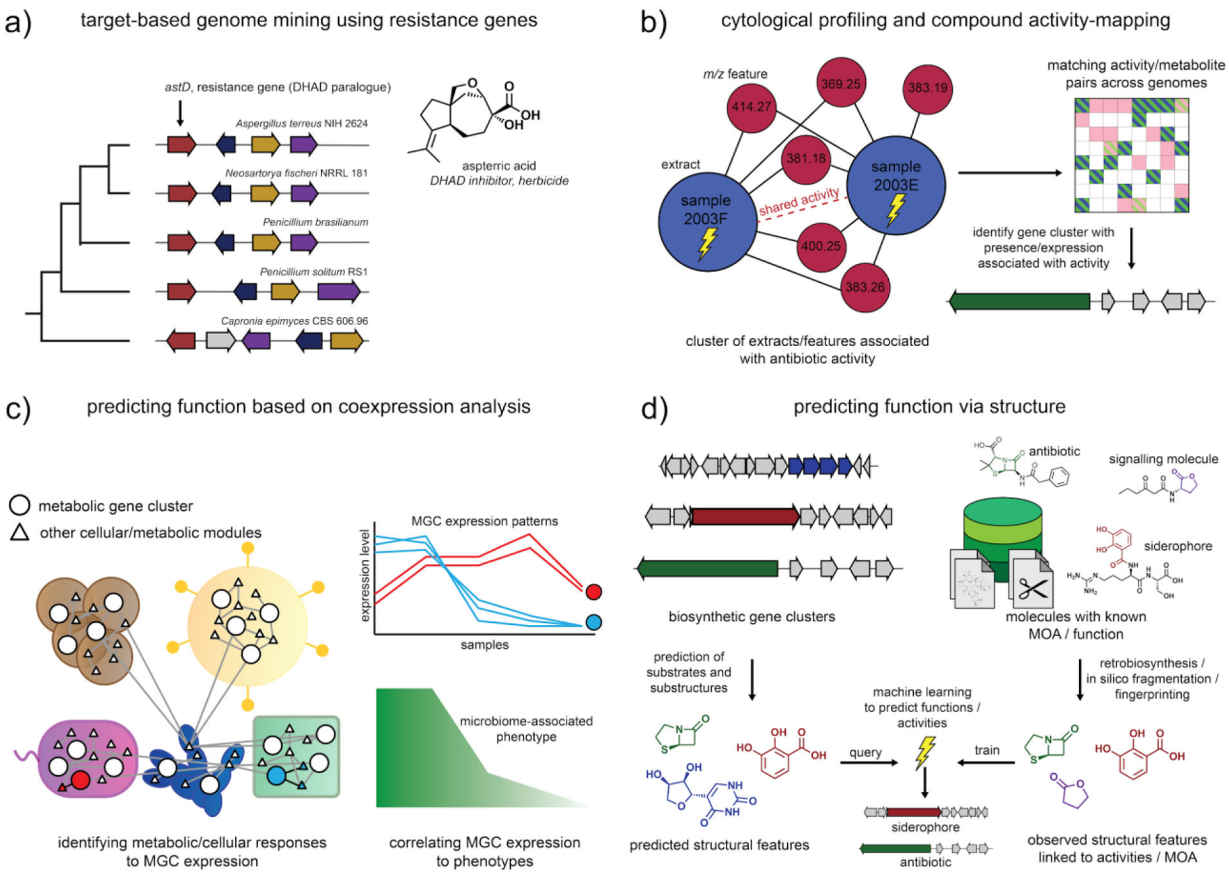

a)
target-based genome mining using resistance genes
astD, resistance gene (DHAD paralogue)
Aspergillus terreus NIH 2624
Neosartorya fischeri NRRL 181
Penicillium brasilianum
Penicillium solitum RS1
Capronia epimyces CBS 606.96
O
O
OH
OH
aspterric acid
DHAD inhibitor, herbicide
b)
cytological profiling and compound activity-mapping
m/z feature
414.27
369.25
383.19
381.18
extract
sample 2003F
sample 2003E
shared activity
400.25
383.26
matching activity/metabolite pairs across genomes
identify gene cluster with presence/expression associated with activity
cluster of extracts/features associated with antibiotic activity
c)
predicting function based on coexpression analysis
metabolic gene cluster
other cellular/metabolic modules
MGC expression patterns
expression level
samples
microbiome-associated phenotype
identifying metabolic/cellular responses to MGC expression
correlating MGC expression to phenotypes
d)
predicting function via structure
antibiotic
signalling molecule
siderophore
biosynthetic gene clusters
molecules with known MOA / function
prediction of substrates and substructures
retrobiosynthesis / in silico fragmentation / fingerprinting
machine learning to predict functions / activities
query
train
siderophore
antibiotic
predicted structural features
observed structural features linked to activities / MOA

Figure 4. Function-first genome mining approaches.

In order to more effectively identify molecules with desired activities, function-first genome mining approaches have been and are being developed. a) In target-based genome mining approaches, self-resistance genes are identified that genomically cluster with the biosynthetic genes. Such self-resistance genes are often resistant copies of a housekeeping gene whose protein product is targeted by the metabolite biosynthesized from the pathway. This provides a way to directly predict the mechanism of action for metabolic products of a subset of gene clusters. b) Cytological profiling can be used to identify the effects that metabolic extracts have on certain cell lines, and compound activity-mapping can identify which underlying mass-spectral features are likely responsible for activities that are shared between extracts. The activities and/or metabolites can then be matched to the presence or expression of genes and gene cluster to identify a candidate biosynthetic route towards the underlying molecule. c) Functions of products of biosynthetic genes and gene clusters can be predicted by looking for coexpression with other genes in the same organism (predicting function based on the guilt-by-association principle) or across organisms (identifying the potential effect that a pathway has on other organisms or on a microbiome-associated phenotype). d) Structural features and substructures that are likely part of the metabolic product of a gene cluster can be predicted *in silico*; sometimes, these substructures are diagnostic for a certain mechanism of action or biological activity, and machine learning algorithms can be trained to predict these activities based on sets of structural features.