Activation of microbial secondary metabolic pathways: Avenues and challenges

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ABSTRACT

Microbial natural products are a tremendous source of new bioactive chemical entities for drug discovery. Next generation sequencing has revealed an unprecedented genomic potential for production of secondary metabolites by diverse micro-organisms found in the environment and in the microbiota. Genome mining has further led to the discovery of numerous uncharacterized 'cryptic' metabolic pathways in the classical producers of natural products such as Actinobacteria and fungi. These biosynthetic gene clusters may code for improved biologically active metabolites, but harnessing the full genetic potential has been hindered by the observation that many of the pathways are ‘silent’ under laboratory conditions. Here we provide an overview of the various biotechnological methodologies, which can be divided to pleiotropic, biosynthetic gene cluster specific, and targeted genome-wide approaches that have been developed for the awakening of microbial secondary metabolic pathways.

1. Microbes: promising and prolific source of new drug leads

Natural products (also referred to as secondary metabolites or specialized metabolites; SMs) represent a group of low-molecular weight structurally diverse and complex bioactive compounds occupying an unusual chemical property space [1]. Microbes in particular are prolific antibiotic factories [2], and have proven to be a bountiful source of SMs that have been successfully developed as crucial drug leads [3,4]. To date, more than 5000 antibiotics have been identified from the genus Actinobacteria, while 500 natural products have been isolated from Streptomyces bacteria are particularly profuse producers with 7600 SMs identified until 2005 [8], while computational predictions have estimated that these bacteria may have the capability to produce 150,000 chemically distinct antimicrobial agents [5–7]. In addition to classical antibiotics, microbial cultures have been a source for immune-suppressive agents [9], lantibiotics, anti-proliferative [10], cytotoxic [11], anti-hypertensive [12], antiviral compounds [13,14] and various enzyme inhibitors [15]. Circum 35% of drugs approved by the FDA/EMA are estimated to be either natural products or their derivatives [16], and > 50% of clinical antibiotics are of Actinomycetes origin [8]. Widely used antibiotics include erythromycin A, penicillin G and streptomycin, while examples of microbial metabolites that have been successfully launched for other therapeutic areas comprise the anticancer agent doxorubicin, the immunosuppressants rapamycin and cyclosporine and the anthelmintic drug avermectin B1 (Fig. 1).

The chemical entities described above (Fig. 1) were discovered several decades ago, most during the “Golden Era of Antibiotics” in the 1950s and 1960s. Starting from the 1980s and 1990s, traditional bioactivity-based screening of microbial culture extracts became severely affected by diminishing returns due to high probability of discovering previously known metabolites [17]. Structure elucidation of hit molecules is time consuming and ultimately the high rediscovery rate of known molecules led to decreased interest of pharmaceutical companies in natural products [2], which turned their attention to structure-based drug design and combinatorial chemistry instead [18,19]. However, despite very strong investments into this field in the last 15 years, these efforts have not provided a single novel synthetic antimicrobial agent that has proceeded beyond preliminary clinical trials [20]. One key problem has been that while many synthetic compounds with high efficacies towards their molecular targets have been successfully developed, it has become apparent that these compounds have difficulties in reaching their target site in vivo and in penetrating bacterial cell membranes [17].

However, recent technological advancements have created a renaissance of interest in natural products and new chemical entities with
Fig. 1. Chemical structures of selected microbial natural products.
novel mode of action [21,22]. The foundation was laid in the 2000s by the diligent work of the Academic sector in elucidating the biosynthetic logic of the main classes of microbial natural products made by polyketide synthase (PKS) [23,24], non-ribosomal peptide synthetase (NRPS) [25] and ribosomally synthesized and post-translationally modified peptide (RiPP) pathways [26]. The classical pre-genomics view was that an individual microbial strain could produce only a limited number of SMs, but the first genome sequences of Streptomyces [27] and Aspergillus [28] published in the 2000s and the subsequent explosion of genome sequencing data in the 2010s has demonstrated that these micro-organisms have the genetic capability to produce a significantly larger number of compounds with great strain-to-strain variations. These unknown metabolic pathways are likely to encode numerous bioactive molecules, which could be used to solve the current problems with drug resistant pathogens and to obtain improved chemotherapy agents with reduced side effects. The key question remaining to answer in the field is how to access this hidden potential, since it would appear that the most of these cryptic pathways are silent or poorly expressed under laboratory conditions. In our current discussion, we attempt to provide crucial insights into methodologies that have been developed in an attempt to harness this tremendous chemical diversity.

2. Genomics-driven natural products discovery

The paradigm shift to genomics-based discovery of microbial SMs may be considered to be initiated by the sequencing of the genomes of S. coelicolor and S. avermitilis, which led to the discovery of 22 [27] and 25 [29] putative biosynthetic gene clusters (BGC), respectively, that could code for secondary metabolic pathways. The genome of the filamentous fungi Aspergillus nidulans harbored an even greater potential with 56 putative pathways, as observed by genome mining [30]. However, recent next generation sequencing efforts have vastly expanded this phenomenon in an unparalleled scale to diverse microbial genomes that have not typically been associated with natural products such as Barkholderia, Clostridium and Pseudomonas [31,32]. In addition, probing microbial communities in various ecological niches such as the human microbiota [33] and environmental samples [34] have revealed exceptional metabolic diversity. Many lactic acid bacteria, including species of Enterococcus, Lactobacillus and Streptococcus associated with the gastrointestinal tract have been reported to produce bioactive modified peptides [33], in particular those synthesized via RIPP pathways [35,36]. The marine sponge Theonella swinhoei has been shown to host many uncultivated bacterial symbionts, which harbor pathways for production of exotic SMs [37]. It should also be noted that the potential of soil microbes for production of SMs remains elusive, since ca. 99% of environmental microbes are un-cultured under laboratory settings. In order to circumvent this barrier, environmental DNA isolated directly from soil samples may be used for cloning and expression of BGCs, as in the case of the malacidins pathway [38]. In another approach, the broad spectrum antibiotic teixobactin was discovered from an uncultured soil bacteria 'Eleftheria terrae' by growing the microbe directly in situ in the soil using a bacterial iChip [39].

The development of bioinformatics software for analysis of sequencing data for the presence of BGCs has been instrumental in genomics-driven natural products discovery. Programs such as antiSMASH [40,41], SMURF [42], BAGEL3 [43] and PRISM [44,45] are able to detect and decipher all of the most common biosynthetic types. In some instances, such as type I polyketides and NRPSs and RiPPs, these programs are able to even provide predictions for the chemical scaffolds of the metabolites [45], but for other compound classes such as type II PKSs and glycosylated natural product, the information acquired through computational studies is limited. Linking genomic data to chemistry has also been aided by community standards such as MIBiG (Minimum Information about a Biosynthetic Gene cluster) [46], which define standardized annotation and metadata procedures for characterization of pathways thus facilitating future research efforts.

3. Metabolic gene clusters in microbes

The genome mapping approaches are facilitated by the fact that all genes required for the biosynthesis of the metabolite, its regulation, resistance and transport are typically located in within the BGCs [47–49]. The size of metabolic gene clusters varies greatly depending on the complexity of the end product of the pathway. As an example, the gene cluster responsible for biosynthesis of the glycosylated anthracycline nogalamycin encodes 32 enzymes [50]. In addition, the type of biosynthetic machinery has a significant influence to the size of the BGC with large differences noted between the 30-kb thiostrepton and 128-kb daptomycin gene clusters [51,52], even though both are assembled from amino acid building blocks and are of similar complexity. The difference can be explained with the fact that thiostrepton is RIPP [51], whereas daptomycin is made through a NRPS pathway. Wide variation may also be found within the same classes of biosynthetic machineries, since the chemically simple NRPS showdomyacin is encoded within a 12-kb fragment of DNA, while the pristinamycin supercluster, which encodes two complex hybrid PK-NRP molecules, spans as large as 210-kb [53].

The onset of SMs production typically occurs during the early stationary phase, and involves complex metabolic changes within the organism [54]. Pleiotropic (or global) regulators are localized distantly from the biosynthetic cluster, and control the expression of secondary metabolism by responding to diverse signals [55]. These pleiotropic regulators have wider coverage, simultaneously controlling the expression of several BGCs. Events of nitrogen or phosphate starvation or distinct signaling compounds like N-acetylglucosamine (GlcNAc), stressors like heat, pH and damage to the cell wall provide proper stimuli for triggering the expression of these regulatory genes [56,57]. The production is also guided by regulatory genes present in the biosynthetic clusters, which are pathway-specific and may either be activators or repressors [58].

The genes responsible for providing resistance and for transporting SMs outside of the cells are also typically clustered within BGCs. In particular, if the end product of the pathway has biological activity against the producing host, then the resistance genes for that particular metabolite may be encoded in the genomic locus. Similarly, genes encoding efflux pumps (for instance, ATP-binding cassette (ABC) and Major Facilitator Superfamily proteins) have also been traced along the BGCs [59] and are responsible for the reduction of both toxicity [60] and feedback inhibition effects. For instance, in S. avermitilis, the ABC transporter AvtAB responsible for the exporting avermectin is found within the biosynthesis gene cluster [61].

4. Strategies for the activation of unknown metabolic pathways

A growing body of evidence has indicated that the activation of gene clusters has the potential to greatly facilitate discovery of new natural products of high-therapeutic leads. Several methods have been developed for the activation of silent or poorly expressed cryptic gene clusters, which can be broadly classified to three major categories (Fig. 2). In one group, there are methodologies that aim to modify the whole metabolome of the target strain and generate pleiotropic effects to activate randomly any pathway residing in the strain. These methods are generally technically simple and are therefore suitable for scaling up to high-throughput systems. Unfortunately, these techniques generally forfeit the advantages provided by next-generation sequencing and genome mining, since they are not able to target the most interesting gene clusters for activation. They also suffer from the same metabolite rediscovery issues as traditional natural products discovery. In contrast, the methodologies lying under the second category are able to focus on desired pathways, but are technically challenging and suffer from low throughput. Finally, the techniques in the third group aim to
Fig. 2. Methodologies for the activation of silent gene clusters to obtain new natural products. Petals within the circle represent different techniques that have been developed with three major categories, viz., pleiotropic, BGC-specific and targeted-genome wide approaches highlighted. Abbreviations: OSMAC, One strain many compounds; P, Promoters; PPTases, Phosphopantetheinyl transferases; RGMS, Reporter-Guided Mutant Selection.
gain the benefits of both of the approaches mentioned above, usually by combining pleiotropic activation methods to gene cluster specific reporter systems. The diversity of these methodologies offer many tools for the research community, but to date no single superior method for activation of biosynthetic pathways has been presented.

4.1. Genome-wide pleiotropic methods

4.1.1. Ribosome engineering and its applicability

One of the most important adaptations in bacteria to environmental stresses, e.g. depletion of amino acids, is the “stringent response” governed by an intracellular transient accumulation of guanosine tetraphosphate (ppGpp) [62]. The bacterial alarmone ppGpp is synthesized by the ribosomal enzyme ppGpp synthetase (relA) and is initiated by the binding of an uncharged tRNA molecule to the aminoacyl binding site (A-site) on the ribosome [63]. Subsequent binding of ppGpp to the RNA polymerase (RNAP) modulates transcription by upregulating and downregulating various promoters [64,65] through mechanisms that can vary between species [66]. Investigations into the RNA polymerase/ppGpp complex in a thermophilic bacterium Thermus thermophilus revealed three modes of transcriptional regulation by ppGpp depending on the nucleic acid composition of the promoter, which influenced the conformation of the RNAP [64]. Therefore it is unsurprising that ppGpp has also been linked to secondary metabolism and initiation of antibiotic production [67]. In Streptomyces, relA/relC mutants with significantly decreased ppGpp contents display lowered production of SMs when transferred to a media with nutritional shift-down [68–70] (Fig. 3A and B). It has been revealed that mutations conferring resistance to rifampicin in the rpoB gene, which encodes the RNA polymerase β-subunit, alter the conformation of the RNAP to resemble ppGpp bound status. Therefore, the requirement for ppGpp for the expression of silent or poorly expressed gene clusters is bypassed in these mutants in various actinomycetes [71–73].

In an analogous manner, the acquisition of mutations in ribosomal proteins conferring resistance to antibiotics targeting the ribosome has also been reported to promote the expression of silent genes. However, the molecular mechanism of this phenomena remains obscure. The introduction of streptomycin-resistance mutations in rel mutants of S. coelicolor and S. griseus reinstated actinorhodin production without the requirement of ppGpp [74,75]. On the contrary, elevated concentrations of ppGpp (30-fold) and actinorhodin (180-fold) has been detected in S. coelicolor, when the strain was exposed to eight antibiotics in a sub-inhibitory concentration [76]. In addition, relA disruption resulted in the total loss of ppGpp accumulation as well as a significant decrease in actinorhodin production, indicating the key role of ppGpp in the activation of biosynthesis. Cluster-activated strains resulting from mutations in the ribosome have been reported in Streptomyces and other bacteria including Bacillus spp., Mycobacterium spp. and Pseudomonas spp. [74,77,78]. Strikingly, two single rpoB mutants, an rpoB + rpsL double mutant, and a single gentamicin-resistant mutant induced the production of piperidincams in S. maevicolor, whereas the wild-type does not produce these metabolites at any detectable level in various media [79].

4.1.2. Chromatin remodeling

Chromatin remodeling is a vital mechanism for the modulation of transcription of eukaryotic genomes [80]. Chromatin is composed of repeating nucleosome units, which consist of histones (2 copies each of H2A, and H2B, H3 and H4 [80]) wrapped around chromosomal DNA and linked together through a linker histone (H1) (Fig. 3C) [81,82]. Histones cause dynamic variations to packaging and accessibility of DNA in response to physiological and developmental cues [83,84]. These are mediated via post-translational modifications such as deacetylation and methylation of histone tails, which ultimately regulate gene expression [85]. Gene clusters for secondary metabolism are clustered at subtelomeric regions and are co-regulated [86], where histone acetylation and methylation largely impact transcription [87].

For activation of SMs, the state of chromatin packaging has been influenced by artificial expression of histone modification genes and by utilizing small molecule inhibitors against histone deacetylase enzymes. In A. nidulans, inactivation of the histone deacetylase (HDAC) hdaA down-regulated secondary metabolic pathways [88], while loss-of-function of cclA (an ortholog of bre2 involved in histone H3 lysine 4 methylation) activated the expression of the cryptic secondary metabolic clusters generating monodictyphenone, emodin and its derivatives [89]. The use of the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) led to stimulation of production of new cladochromes and calphostin B in Cladosporium cladosporioides [90] and histone deacetylase (HDAC) inhibitors have also caused over-expression of SM genes in A. nidulans [88,91].

Unlike in fungi and other eukaryotes, prokaryotes are entirely deprived of histones. However, the bacterial genome, for instance, Streptomyces sp. comprises their own versions of HDAC [92] and the structure of the nucleoid may have a role in the global regulation of metabolism [93]. Access to chromosomal DNA may be restricted by nucleoid-associated proteins, various RNAs and differential supercoiling in the nucleoid, which may prevent aberrant transcription of many gene clusters [94]. In Streptomyces, a bifunctional nucleoid-associated protein (DdbA) has also been characterized that comprises of an N-terminal DNA-binding histone H1-like domain and a C-terminal DksA-like domain, which could modulate the RNA polymerase activity along with ppGpp to induce transcription [95].

4.1.3. OSMAC approach and environmental cues

One strain many compounds “OSMAC” is a relatively simple and versatile approach that allows activation of diverse-metabolic pathways [96]. The biosynthesis of SMs is stimulated through alterations in cultivation parameters [97,98], e.g. media composition, aeration rate, type of cultivating vessel or a combination of these factors. In some cases, even subtle changes may result in drastic changes, as demonstrated in fermentations of Paraphaeosphaeria quadripertata, where switching from the use of tap water to distilled water lead to the isolation of six new SMs [99]. These experiments aim to mimic more precisely the natural growth conditions found in the environment to trigger the onset of secondary metabolism, and has been successfully applied to both bacteria and fungi, as reviewed elsewhere [97,98].

The classical OSMAC approach may be extended to more drastic measures by modulating the culture conditions with stress or chemicals. Heat shock has been shown to induce jadomycin production [100] and increase the yields of validamycin [101], whereas limitation of nutrients such as alanine and/or an acidic pH shock led to methylenomycin production in Streptomyces coelicolor A3(2) [102]. In particular, numerous SMs have been shown to be regulated negatively by inorganic phosphate concentrations [57], while GlcNAc appears to function as an important sensory molecule for the onset of secondary metabolism [103]. Both biotic (microbial lysates or soil extracts) and abiotic (antibiotics, synthetic compounds) chemical elicitors have been proved to be effective strategies for the induction of silent gene clusters. Two small molecule elicitors have been particularly successful; AR2C2, a synthetic compound discovered through large scale screening [104], and goadsporin [105,106], a natural product that promotes secondary metabolism in Streptomyces. Sub-inhibitory concentrations of other xenobiotic antibiotics, such as jadomycin B [107] and monensin [108] have been shown to induce endogenous production of SMs. Interestingly, it has been shown that the addition of rare earth elements, e.g. scandium and lanthanum, at low concentrations resulted in the activation of the cryptic secondary metabolic biosynthetic gene clusters [78,109].

Co-cultivation, mimicking the ecological habitats of microorganisms, has been emerged as an effective strategy to increase the chemical diversity of secondary metabolites (Fig. 4A). It is believed that the
social networking in ecological environment is mediated by diffusible low-molecular-weight signaling molecules that are secreted from and received by cells [110,111]. For example, co-cultivation of the marine-derived fungal isolate A. fumigatus MR2012 with S. leeuwenhoekii strain C34 led to induction of metabolite production in both organisms, while co-cultivation of the fungus with bacterial strain C58 resulted in the detection of pentalenic acid from the bacteria [112]. Another marine-derived fungus Emericella sp. was induced to produce emericellamides A and B upon co-culture with Salinispora arenicola [113], whereas pro-momycin produced by one Streptomyces strain led to initiation of antibiotic production in another strain by cross-feeding [114]. Interestingly, dialysis experiments and electron microscopy have revealed that not only diffusible signals, but also intimate physical interactions contribute to the networking and induction of silent clusters during co-cultures of A. nidulans with S. hygroscopicus [115] or A. fumigatus with S. rapamycinicus [116].

4.1.4. Phosphopantheteine transferases

The biosynthesis of polyketides and nonribosomal peptides only occurs if the essential acyl - and peptidyl carrier proteins, respectively,
Fig. 4. Pleiotropic approaches for the activation of the silent gene clusters: Global regulators, PPTases and the OSMAC approach. A) In the OSMAC approach, cultivation conditions are manipulated to uncover the environmental signal required for activation of biosynthesis. As an example, co-cultivation of two or more microbes may induce microbial chemical communication, where one or even both participants produce novel compounds. Abbreviations: A, Activator; BGC, Biosynthetic gene cluster; P, Promoter. B) PPTases catalyze the post-translational modification of carrier proteins, which is essential for the biosynthesis of polyketides and nonribosomal peptides. Overexpression of PPTases may influence the production of SMs under conditions where the native PPTase may be downregulated. Abbreviations: A, Adenylation domain; ACP, Acyl-carrier protein; AT, Acyltransferase; C, Condensation domain; KS, Ketosynthase domain; NRPS, Nonribosomal peptide synthetase; PCP, Peptidyl-carrier protein; PKS, Polyketide synthases; TE, Thioesterase domain. C) Pleiotropic regulators control many aspects in the life cycle of microbes. Manipulation of these regulatory networks may lead to the activation of BGCs.
are post-translationally modified into active holo-form [117]. This re-
action is catalyzed by phosphopantetheinyl transferases (PTases) through covalent attachment of a 4'-phosphopantetheine group to a
highly conserved serine residue (Fig. 4B). Microbial genomes may
contain up to three PTases, which harbor specificity to various bio-
synthetic pathways [118], but these genes are typically not found
within BGCs. Since PTases regulate primary metabolism that leads to
physiological changes and metabolic flux alterations, overexpression of
PTases may also influence secondary metabolism [119]. Indeed, ex-
pression of two PTase genes spf and ssp resulted in activation of silent
pathways with a success rate of 70% in a study using 33 Actinomycetes
strains [120]. The spf and ssp gene products, from Bacillus subtilis and
Streptomyces verticillus, respectively, exhibit broad substrate specificity
to guarantee sufficient phosphopantetheinylation of diverse biosyn-
thetic enzymes. Recently, PTase-based activation also led to the iden-
tification of three nucleosides including puromycin A and two new
congeners in S. alboniger NRRL B-1832 [121]. Interestingly, the bio-
synthetic pathways of nucleosides do not contain carrier proteins,
suggesting that the result was achieved indirectly via modulation of
cellular regulatory networks by the PTase.

4.1.5. Manipulation of global regulatory systems

The engineering of global regulatory genes aims to influence the
same regulatory networks as the OSMAC approach, but instead of
trying to find the environmental cues, it does it at a more refined mo-
lecular level through over-expression or inactivation of the genes con-
trolling the cellular response to these phenomena (Fig. 4C). Investiga-
tions into pleiotropic regulatory networks in bacteria and fungi have
revealed that production of SMs is linked to morphological develop-
ment and nutrient availability (carbon and nitrogen sources, phosphate
starvation, amino acid and iron availability) [55]. Other examples in-
clude response to pH stress in fungi mediated by the FadC zinc finger transcription factor [122] and the master regulator DasR in Strepto-
myces, which responds to the presence of N-acetylglucosamine that may
be considered as a signal for autolytic degradation of the vegetative
mycelium [103]. An important distinction to cluster situated regulatory
genes is that the global regulatory systems also control the transcription of
many other genes not related to secondary metabolism. In fungi,
pathway-specific regulatory genes have been discovered only in about
60% BGCs [123], which indicates that the production of many SMs is
modulated strictly by global regulatory networks. Similarly, the mole-
necyin biosynthetic gene cluster does not harbor a pathway-specific
regulator and is under the control of pleiotropic regulators in Strep-
tomyces ghanaensis [124].

In Streptomyces, manipulation of regulatory elements that respond to
butyrolactone hormones such as adaA [125] have been particularly
successful with cryptic ovidomycin [126] and germicidin [127] gene
clusters recently activated. Disruption of whiB-like regulatory genes,
which are associated with control of morphological development [128],
have also led to the discovery novel violaplyrones [129] and antymycin-
type depsipeptides [130].

4.2. Biosynthetic gene cluster specific approaches

4.2.1. Transcription factors and promoter exchange experiments

In eukaryotes, each gene is transcribed from its own promoter,
whereas in prokaryotes (e.g., Streptomyces sp.) several genes are often
clustered together to form operons (Fig. 5A). In all bacterial species, the
promoter sequences residing within BGCs are typically associated with
specific sigma factors (σ-factors) that are vital for binding of the RNA
polymerase (RNP) in the formation of the translation initiation com-
plex [131]. The various σ-factors confer promoter selectivity and are
thereby responsible for switching on specific regulons [132]. For in-
stance, the filamentous prokaryotes, S. albus J1074, S. coelicolor, and S.
avermitilis comprise 35, 65 and 60 σ-factors, respectively [27,133]. On
the other hand, promoter recognition in fungi relies on more diverse
transcription factors. Eighty families of transcription factors were re-
vealed through a whole-genome analysis of more than 200 fungal
organisms [134]. For example, the filamentous fungi A. nidulans and N.
crasa contain 81 and 58 transcription factors, respectively [135]. These
aforementioned mechanisms enable alterations in transcription pat-
terns in response to environmental cues.

The σ-factors influence the expression of larger groups of genes,
therefore σ-factor-based engineering methods have been utilized to
activate gene expressions associated with secondary metabolite bio-
synthesis. For example, overexpression of the σ-factor ‘Orf21’ in
Streptomyces clavuligerus NRRL3585 resulted in a slight increase of
clavulanic acid production through the induction of early biosynthetic
genes (cesA2, cas2) and the activator gene cacA [136]. Moreover, the
overexpression of σ-factors led to the accumulation of higher level of
FKS06, a hybrid polyketide-norribosomal and carotenoid produc-
dresses in Streptomyces sp. KCCM 11116P [137] and Corynebacterium
glutamicum, respectively [138]. Successful expression of a polyketide
biosynthetic pathway (oxytetracycline) was achieved for the first time
in a surrogate host E. coli by overexpressing the host σE-factor [139].
The deletion of σ-factor ‘SigG’ caused over-production of avermectin
by 2–2.7 fold in S. avermitilis, while overexpression of an extra copy lead to
significant decrease in yields [140]. In addition, S. avermitilis hrdB
mutants lacking σE-abd exhibited higher levels of avermectin produc-
tion than the parental strain [141].

The promoter sequences residing in BGCs do not typically promote
transcription in all growth conditions and are highly influenced by in-
tracellular regulation networks. Manipulation of promoter sequences by
exchanging native silent promoters with functionally active ones di-
rectly in the producing organism has proven to be a useful strategy to
drive expression of the silent genes by circumventing the complexity of
the regulatory genes (Fig. 5B). The methodology requires advanced
gene editing tools that might not be available for the target organ-
isms and is best suited for small BGCs that are composed of very few
operons. The activation of a silent polycyclic tetramate macrolactam
(PTM) gene cluster in Streptomyces albus has been achieved by the in-
dependent introduction of ermE* upstream of fdA and fdb encoding
putative desaturase and hybrid polyketide synthase-norribosomal
peptide synthetase, respectively, and led to the identification of 6-epi-
alteramides A and B. Furthermore, the biosynthesis of a blue pigment
‘indigoidine’, which is not typically produced in standard laboratory
conditions, has been induced in S. albus by expressing the NRPS gene
bpsA under ermE* promoter [142]. The introduction of strong en-
geineered promoter kasO* also resulted in successful activation of silent
lycopene biosynthetic cluster in S. avermitilis [143]. Recently, replace-
ment of the native promoters of the silent actinorhodin, undecylprodigiosin and PTM clusters with kasO* produced targeted
compounds in S. albus, S. lividans and S. roseosporus, respectively [144].

In Aspergillus nidulans, replacing the native promoter of the non-
rribosomalpeptide synthetase, acVA, with a promoter from the ethanol
dehydrogenase, alcA, from the same host led to a 30-fold increase in
penicillin production [145]. In addition, sequential exchange of pro-
moters for six genes in the silent fellutamidine B gene cluster (inp) with
alcA enabled the activation of the cluster and led to the production of
the target compound in A. nidulans [146].

4.2.2. Manipulation of pathway-specific regulatory genes

Pathway-specific transcriptional regulatory genes are typically
found within the BGCs and they may either repress or activate
the biosynthesis of the corresponding SM. These genes represent the lowest
organizational level in the cellular regulatory networks and are typi-
cally tightly regulated by environmental sensors and pleiotropic reg-
ulators. However, in reality the regulatory circuits are likely to be even
more complex with cross-talk between distinct pathways [147] and
even between global and BGC specific regulatory genes reported [148].
The co-localization of pathway-specific regulatory genes offers a po-
sibility for targeted activation of the desired BGC either by inactivation
of the repressor or over-expression of the activator genes (Fig. 5C and D). The caveat is that for many families of transcriptional regulators it is not possible to identify whether the gene is a repressor or an activator based on sequence information alone. The diversity of microbial regulatory proteins is expansive [149], and only two systems are described here to demonstrate the functional differences.

The TetR family repressors consist of a helix-turn-helix DNA binding domain and a receptor domain that responds to the presence of a small molecule ligand [150]. In the absence of the specific signaling molecule, TetR dimers bind to DNA and prevent transcription of downstream genes. Binding of the ligand induces a conformational shift that repositions the helix-turn-helix motifs of the dimer far apart in such a way that the proteins lose their ability to bind DNA [151]. Interestingly, some TetR proteins have been proven to respond to γ-butyrolactone.
autoinducers during the onset of secondary metabolism [152], while others bind directly to both endogenous and exogenous SMs [107,153]. Inactivation of TetR family repressors have led to the activation of ja-
domycin [153,154], kinamycin [155], auricin [156] and ceoelomycin biosynthesis [157,158].

Another predominant class of regulators is homologous to LuxR from Vibrion fischeri, which is a transcription factor involved in quorum sensing [159]. The protein consists of an α/β/α sandwich domain which binds the activator ligand and a DNA binding response domain. In contrast to the TetR family, most characterized LuxR proteins are transcriptional activators, where ligand binding induces homodimerization, which enables binding of the protein to DNA in so-called tra box sequences and initiate transcription [160]. An important sub-family of LuxR regulators in Actinobacteria are LAL-proteins (Large ATP-binding regulators of the LuxR family) [161], originally discovered in the region for maltose utilization, that contains an additional ATP-binding domain at the N-terminus responsible for the interaction with the inducers, maltotriose and ATP [162]. In a recent example, LuxR-type activators were over-expressed and TetR-type repressors were inactivated to initiate production of totopotensamides [163].

4.2.3. Heterologous expression in surrogate hosts

Many microbes that have the capability to produce natural products, such as strains of Cyanobacteria and filamentous fungi, are genetically intractable [164]. Heterologous expression provides an opportunity to access the genomic potential these organisms harbor in a genetically compliant host. The capture of target BGCs was classically done through screening of cosmids gene libraries, which was both time-consuming and could not accurately clone the desired DNA fragment, but advances in direct cloning strategies and DNA assembly tools have provided alternative methods. These methodologies are described in more detail in Section 4.2.4., since changing the expression host may require manipulation of control elements such as promoter sequences to ensure sufficient transcription levels (Fig. 5E).

The choice of expression host is far from trivial and many factors need to be considered. The genetically malleable Escherichia coli and Saccharomyces cerevisiae provide attractive hosts for production of bacterial and fungal SMs, respectively, but since neither is naturally equipped for production of complex chemicals, issues with metabolic flux, codon usage, self-resistance and activation of biosynthetic acyl/peptidyl carrier proteins need to be resolved. E. coli seems to be well-suited for expression of pathways originating from Cyanobacteria that are challenging to modify genetically, with production of microcystin [165] and lyngbyatoxin reported [166]. Several fungal terpene syntheses have also been expressed in E. coli, but multi-gene clusters are much more challenging due to the requirement to remove introns and modify promoter regions. E. coli has had less success with BGCs originating from high-GC Actinobacteria, even though the entire erythromycin pathways has been expressed in this host [167-169]. The titres of SMs produced in E. coli have been generally low. Other promising heterologous hosts for production of prokaryotic SMs include Myxococcus xanthus, Pseudomonas putida [170] and Bacillus subtilis [171].

In many instances, it would appear that hosts that are evolutionarily closely linked to the organism harbouring the target pathway may be best suited for heterologous expression. Several well-characterized Streptomyces sp. are easily amenable and are efficient surrogate hosts for the heterologous characterization of different genes from Actinobacteria. In particular, S. albus J1074 [172], S. lividans TK24 [173], S. venezuelae ATCC10712 and S. coelicolor A3(2) have been widely used for the expression of cryptic pathways. S. coelicolor is genetically the most studied actinomycete and a large array of tools are available for manipulation of the organism [174], whereas the closely related S. lividans TK24 has particularly excelled at expression of proteins [175]. S. venezuelae, is well-suited for the heterologous expression of the PKs and NRPS due to the presence of large number of innate PPTase genes [176]. Finally, cloning genes in S. albus is facilitated by a defective restriction modification system and the naturally minimized genome may help increasing product titres [177]. In filamentous fungi, Aspergillus species have been widely used as heterologous hosts. An early example includes the transfer of the entire penicillin gene cluster to A. nidulans, while more recently geosmin has been heterologously produced in this host [178-180]. A. oryzae has gained interest for heterologous expression, as it has traditionally been used in the food industry and has been given the status of GRAS (generally regarded as safe) organism. Selected recent examples of SMs produced in this host include paclixilone [181], afatem [182] and pleuromutilin [183]. The heterologous expression of A. terreus asperfuranon cluster in the model ascomycete, A. nidulans, resulted in awakening of the asperfurane biosynthetic pathway when several non-reducing polypeptide synthase genes were placed under the control of diACP [184].

An ideal host for heterologous expression would provide sufficient metabolic building blocks for the synthesis of SMs, but would not have native pathways that could interfere with the biosynthesis or generate background. To this end, several strains have been genome minimized to function better as expression chasses. Four gene clusters have been deleted from the genomes of S. coelicolor M1152 and M1154, which have been further improved for metabolite production by ribosome engineering [185]. Extensive manipulation, including deletion of a 1.5 Mb subtelomeric region and 78% of putative transposase genes of wild type S. avermitilis led to high success rates and product yields in the expression of exogenous biosynthetic pathways [186]. These ongoing efforts are likely to result in a handful of “superhosts” for the heterologous expression of diverse metabolic pathways.

4.2.4. Refactoring of gene clusters

The emergence of new metabolic engineering methods and the continually decreasing costs of DNA synthesis have enabled complete refactoring of entire BGCs, which typically includes three distinct stages. The first step involves the architectural design of the synthetic gene cluster from biological part libraries to obtain desired promoter, gene and terminator sequences, which may be ordered synthetically or amplified using PCR. In the next step, the pathway is assembled together with advanced cloning methods such as recombinerring or TAR-cloning typically in E. coli or Sac. cerevisiae. TAR cloning takes advantage of the high efficiency natural homologous recombination in yeast that allows for both direct capture of BGCs and simultaneous cloning of multiple DNA fragments [187]. In contrast, recombinerring utilizes recombination systems from phages to enable homologous recombination to occur in E. coli [188]. In addition, the combination of long-amplicon PCR and DNA recombination (DiPacC) has recently been utilized for the direct cloning of the anaabaopeptins and erythromycin pathways [189]. The ultimate step involves the transfer of the artificial BGC to the expression host selected for the production of SMs (Fig. 5F).

Reports on fully synthetic refactoring of BGCs have still been scarce due to the significant costs associated with the method. Even though this approach would allow for unprecedented freedom in pathway design, the complexity of metabolic pathways required for the production of even simple SMs makes the task of designing functional BGC in silico far from trivial. For these reasons, the methodology appears to be best suited for improvements in the production titres of known value-added metabolites and not for the activation of unknown pathways. Examples of fully synthetic refactored gene clusters include rebeckamycin and pyrrolnidin [190].

A more widely used approach in refactoring has been to modify regulatory elements or promoter regions of natural gene clusters prior to heterologous expression. Expression of the unmodified taromycin gene cluster directly in S. coelicolor M1146 did not lead to the production of the lipopeptide, but activation of the pathway was achieved by deletion of pathway-specific repressors using in vivo yeast recombination-mediated PCR targeting [191]. Conversely, refactoring of a strong constitutive promoter in front of a cluster situated activator
enabled the heterologous production of pacidamycin D \[190\]. In a more arduous approach, individual genes or short operons from BGCs may also be amplified by PCR and placed in front of active promoters. After reassembly of full length pathways, polycyclic tetramate macroalactams \[192, 193\] and spectinabilin \[194\] have been produced in heterologous hosts. The PCR–amplification step was circumvented in the refactoring of the novobiocin gene cluster, where a cosmid harbouring the BGC was modified using recombineering to facilitate the generation of plug-and-play expression modules \[195\]. Through a plug-and-play promoter insertion strategy, introduction of three novel constitutive promoters of \(S. coelicolor\), upstream of \(jadJ\) in the cryptic jadomycin B gene cluster in \(S. venezuelae\), led to cluster activation. Importantly, expression of \(jadJ\)
from these three promoters led to higher transcription levels than when the widely used ermFp* was utilized [196]. The benefit of strong constitutive promoters is that they may lead to increased transcription levels and product yields, as has been shown in the engineering of polycyclic tetramet macrolactams [193].

4.3. Targeted genome-wide methodologies

To date, only few approaches have been developed that are able to focus on the desired cryptic BGC detected by genome mining, which are able to utilize pleiotropic methods to widely probe conditions that lead to activation of the pathway. These targeted genome-wide methodologies (Fig. 6) typically take advantage of a biosensor to report increased transcription or other activity from the desired BGC, but differ in the ways of generating alterations to the global transcriptome.

4.3.1. Reporter guided systems

A pioneering Reporter-Guided Mutant Selection (RGMS) method developed in the group of Prof. Keqian Yang provides a widely applicable approach for targeted activation of silent gene clusters in the native organism [197]. RGMS combines two effective yet simple techniques: cloning of key promoter sequences from the gene cluster of interest in front of a double xylE-neo reporter system and the use of genome-wide mutagenesis to generate a library of mutants that contain tremendous genomic diversity. The core rationale relies on randomly modulating the complex regulatory cascades that are controlling secondary metabolism and converting expression of the target pathway into a signal that enables selection of a mutant with the desired phenotype. The reporter cassette allows initial selection by kanamycin resistance (neo), followed by colour-based detection of catechol oxidase (xylE) activity, which allows for distinction between spontaneous kanamycin-resistant and genuine target-activated mutants (Fig. 6A).

RGMS was initially developed for industrial strain improvement purposes by taking advantage of the correlation between increased transcription levels from the target BGC and product yields. The method has been used to improve production of lovastatin [198], clavulanic acid [199] and natamycin [200] in Aspergillus terreus, S. clavuligerus and S. gibbosa, respectively. In addition, the methodology has also been successfully carried out in combination with ribosome engineering, through exposure to streptomycin, to achieve over-production of daptomycin in S. roseoportus NRRL11379 [201]. Recently, BGC situated TetR–family transcriptional repressors have been developed as alternative biosensors to aid screening of mutant libraries. These reporter proteins bind end products of the pathways and are therefore able to report increased production of the target metabolites directly, which reduces the number of false positives encountered with reporters that only monitor transcription levels. The method was utilized for increasing the production of panamycins in S. alboniger [202].

Many of the cryptic gene clusters denoted “silent” may, in effect, be transcribed at a low basal level, but in such low quantities that the corresponding metabolite remains undetected. Therefore RGMS is also suitable for the activation of BGCs and has been successfully utilized on the jadomycin and gaudimycin pathways in S. venezuelae ISP5230 and Streptomyces sp. PGA64, respectively [197]. The jadomycin BGC is silent under typical culture conditions unless special environmental cues such as heat-shock are applied [203]. To establish proof of concept, jadomycin biosynthesis could be re-activated by using RGMS strategy, whereas work on the cryptic gaudimycin pathway led to the isolation of two novel anthraquinone aminoglycosides [197]. The indigoindole synthetase gene bpsA, which is responsible for synthesis of a blue pigment ‘indigoindigo’, has been also utilized as a reporter in the activation of the cryptic coelomycin gene cluster in S. lividans, where the activation was achieved through over-expression of a pathway-specific regulatory gene [204].

4.3.2. High-throughput elicitor screening

Many SMs are produced in response to various chemical signals, which may either be communication molecules or chemical weapons made by other organisms cohabiting the same environment. If the environmental signal can be identified in the laboratory using small molecule libraries, expression of silent BGCs can be achieved (Fig. 6B). The chemogenetic HITES (high-throughput elicitor screening) method does this by coupling the screening to a promoter-probe system, where either lacZ [205] or egFP [206] is utilized to report increased transcription from the target BGC. The approach has been successfully applied to two gene clusters in the Gram-negative pathogenic model bacterium Burkholderia thailandensis E264, where a silent virulence factor, namely malleilactone (mal) and a poorly expressed burkholdake (bhc) cluster were targeted [205]. The strategy has also been implemented in S. albus J1074, where the nonribosomal peptide surugamide (sur) cluster was awakened [207]. Interestingly, the cytotoxins etoposide and ivermectin were found to be effective inducers resulting the occurrence of 14 novel compounds in the culture media.

5. Concluding thoughts and future perspectives

The abundance of silent and cryptic pathways in microbial genomes provides an exciting opportunity for the discovery of new chemical entities with high therapeutic potential. The diverse strategies developed for awakening these pathways offer an excellent starting point, but to date no single superior methodology has been developed. This is not unexpected, since the BGCs reside in vastly diverse micro-organisms and in many cases are strictly regulated to be activated only under highly specific conditions. The availability of the numerous methods described above, which all have their strengths and weaknesses, can therefore be considered as a significant advantage and it will remain the responsibility of the researcher to choose the most appropriate one for any given project.

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