Minireview

Small-molecule elicitation of microbial secondary metabolites

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Summary

Microbial natural products continue to be an unparalleled resource for pharmaceutical lead discovery, but the rediscovery rate is high. Bacterial and fungal sequencing studies indicate that the biosynthetic potential of many strains is much greater than that observed by fermentation. Prodding the expression of such silent (cryptic) pathways will allow us to maximize the chemical diversity available from microorganisms. Cryptic metabolic pathways can be accessed in the laboratory using molecular or cultivation-based approaches. A targeted approach related to cultivation-based methods is the application of small-molecule elicitors to specifically affect transcription of secondary metabolite gene clusters. With the isolation of the novel secondary metabolites lunalides A and B, oxylipins, cladochromes F and G, nigerone A, chaetoglobosin-542, -540 and -510, sphaerolone, dihydrosphaerolone, mutolide and pestalone, and the enhanced production of known secondary metabolites like penicillin and bacitracin, chemical elicitation is proving to be an effective way to augment natural product libraries.

Introduction

Potentially interesting gene clusters, which may encode metabolites that increase competitiveness in natural environments, can remain silent in the unnatural setting of the microbiology laboratory. There are now many examples of bacterial and fungal biosynthetic secondary metabolite gene clusters outnumbering the number of natural products actually synthesized in the laboratory (Gross, 2009) (Fig. 1). These silent (cryptic) gene clusters undoubtedly harbour an enormous reservoir of novel bioactive constituents for drug discovery.

Aspergillus are a rich source of secondary metabolites (Schneider et al., 2008); they can harbour 30–50 distinct polyketide synthase (PKS) and non-ribosomal peptide synthase (NRPS) gene clusters per species (Payne et al., 2006). In Aspergillus niger fewer than 30% of its 31 PKS, 15 NRPS and 9 hybrid PKS-NRPS (HPN) biosynthetic gene clusters are transcribed under a variety of in vitro culture conditions (Fisch et al., 2009). Sequencing of Streptomyces avermitilis, a known producer of avermectin, revealed at least 25 gene clusters for siderophores, spore pigments and secondary metabolites of polyketide or non-ribosomal peptide origin (Ömura et al. 2001). More than 6% of the S. avermitilis genome contains genes for secondary metabolite biosynthesis. Salinispora tropica, a marine actinomycete, devotes a very large portion of its genome, approximately 10%, to natural product assembly (Udwary et al., 2007).

One can explore the products of cryptic metabolic pathways using either molecular techniques (Gross, 2009; Hertweck, 2009; Scherlach and Hertweck, 2009) or cultivation-dependent approaches (Bode et al., 2002). The number of researchers applying cultivation-dependent approaches appears to be increasing since this decades-old method was formalized (Schiewe and Zeeck, 1999; Höfs et al., 2000; Bode et al., 2002). Very small changes in cultivation conditions, media composition, pH, temperature or aeration, for example, can completely shift the metabolic profile of various microorganisms (Bode et al., 2002). Bode’s group isolated more than 100 compounds from more than 25 structural classes from six different microbes by altering culture conditions (Bode et al., 2002). Cultivation-dependent approaches can be categorized as biotic or abiotic, and abiotic can entail either physical or chemical means (Radman et al., 2003). A biotic approach using entire organisms (i.e. co-culture) to stimulate natural product synthesis was recently reviewed (Pettit, 2009).

As an example of a physical method, UV mutagenesis resulted in the synthesis of a new antibiotic, 2,10-dimethyl 4-hydroxy-6-oxo-4-undecen-7-yne, from Aspergillus ochra-
ceus (Awad et al., 2005), and a new 14-membered macrolide, mutolide 1, from Sphaeropsidales sp. (Bode et al., 2000a). When grown as biofilms in agar lined roller bottles, two Bacillus sp. produced antibiotic activity that was not detected when the strains were grown in shake flasks (Yan et al., 2002). The authors suggest that since these are marine epiphytic bacteria, surface attachment may induce secondary metabolism. Under oxygen limitation, an unidentified red pigment was synthesized by Saccharopolyspora erythraea in either shake flask or batch bioreactor cultures (Clark et al., 1995). In contrast, some Streptomyces natural products are only synthesized under high-aeration conditions (Bode et al., 2002). The fungus Sphaeropsisidales sp., which synthesizes the antifungal spirobisnaphthalene cladosporone bisepoxide, made six new spironaphthalenes when grown under static conditions (Bode et al., 2000b). Other examples of physical conditions that alter secondary metabolite profiles include hydrostatic pressure (Bode et al., 2002).

Alterations in the chemical composition of growth media can have profound effects on secondary metabolite profiles. Changing the water used to make the media from tap to distilled resulted in the isolation of six new secondary metabolites from Paraphaosophoephaeria quadrisepata, cytosporones F-I, quadrisepetin A and 5′-hydroxymonocillin III (Paranagama et al., 2007). When grown in a modified malt extract medium containing soluble starch instead of glucose, Gymnascella dankaiensis produces the unusual steroids dankasterone A and B (Amagata et al., 2007). Aspergillus nidulans synthesizes novel prenylated quinolin-2-one alkaloids when grown on rice medium, but not on a variety of other media (Scherlach and Hertweck, 2006).

An offshoot of the cultivation-dependent approach is the rational use of small-molecule modifiers that target control of cryptic natural product pathways. Studies from almost 20 years ago in which such non-nutrient additives, or elicitors, were shown to affect microbial growth rates or ultrastructure (e.g. Akiyama et al., 1992; Benhamou, 1992) contributed to the current and rapidly expanding interest in elicitation of biologically active secondary metabolites. This review summarizes the current state of microbial small-molecule-directed elicitation, including, where known, mechanisms of elicitation at the transcription, translation or enzyme level. Examples are provided of enhanced production of known metabolites, and completely new metabolites.

Fig. 1. Selected fully sequenced prokaryotic and eukaryotic secondary metabolite producers (Gross, 2009). Dark bars show the numbers of gene clusters known to be present in the genome, based on the number of isolated secondary metabolites at the time of sequencing. Light bars show the total number of gene clusters deduced from whole genome sequence analysis (Used with permission from Harald Gross.).
DNA methyltransferase and histone deacetylase inhibitors

Fungal biosynthetic gene clusters are often located in the distal regions of chromosomes (Nierman et al., 2005; Shwab et al., 2007; Fisch et al., 2009). These regions exist in a heterochromatin state whose genes are often transcriptionally controlled by epigenetic regulation such as histone deacetylation and DNA methylation (Jenuwein and Allis, 2001). Chromatin remodelling by DNA methyltransferase and histone deacetylase inhibitors can result in activation of cryptic biosynthetic gene clusters. Selective manipulation of epigenetic targets using small-molecule inhibitors of histone deacetylase and DNA methyltransferase activities leads to enhanced expression of PKS, NRPS and HPN biosynthetic pathways, and production of new secondary metabolites (Shwab et al., 2007; Fox and Howlett, 2008; Williams et al., 2008; Fisch et al., 2009; Henrikson et al., 2009).

Silencing of secondary metabolite gene clusters can be reversed by removing genes important to the establishment of a repressive chromatin configuration (Bok et al., 2009). In A. nidulans, loss of function CcIA (involved in histone H3 lysine 4 methylation) strains allowed for expression of at least two silent gene clusters, one yielding active anthraquinone constituents, and another active polyketides (Bok et al., 2009). Deletion of A. nidulans hda, which encodes a histone deacetylase, causes transcriptional activation of two telomere-proximal gene clusters, and increased levels of their gene products sterigmatocystin and penicillin (Shwab et al., 2007).

Transcriptional activation also occurs with small-molecule epigenetic modifiers. Twelve diverse fungi, including Aspergillus, Cladosporium, Clonostachys, Diatrype and Penicillium, were treated with DNA methyltransferase and histone deacetylase inhibitors and metabolic profiles evaluated by HPLC, MS, 1H-NMR and TLC (Williams et al., 2008). Eleven of the 12 fungi were responsive to one or more epigenetic treatments based on the production of new natural products and/or enhanced accumulation of constitutive secondary metabolites. Stimulation with amphotericin C, cycloheximide, 5-fluorouracil or multiple media types did not result in new or enhanced production of secondary metabolites. Treatment of Alternaria alternata and Penicillium expansum with the histone deacetylase inhibitor trichostatin A resulted in a statistically significant increase in numerous unidentified secondary metabolites in both species (Shwab et al., 2007).

Treatment of a Diatrype sp. with the DNA methyltransferase inhibitor 5-azacytidine led to the production of two new polyketides, lunalides A and B (Williams et al., 2008) (Fig. 2). Exposure of Cladosporium cladosporioides to 5-azacytidine or the histone deacetylase inhibitor sub-
eryylanilide hydroxamic acid elicited the de novo production of several oxylipins in C. cladosporioides (Fig. 2). Suberyylanilide hydroxamic acid induced two new perylenequinones, cladochromes F and G (Fig. 2), four known cladochromes, A, B, D and E, and calphostin B in C. cladosporioides. According to the authors, this was the first reported co-occurrence of such an extensive range of cladochrome-calphostin metabolites from a single source. Elicitation of cladochromes A and B is especially noteworthy because these compounds were first reported as the unique products of Cladosporium–Cucumis infection of Cucumis sativus seedlings, which were not obtained from a large number of monoculture fermentations (Overeem et al., 1967; Arnone et al., 1988). The small-molecule elicitation results eliminated the possibility that the compounds are the products of mixed Cladosporium–Cucumis biosynthesis.

More than 70% of the 31 PKS-, 15 NRPS- and 9 HPN-encoding gene clusters in A. niger are transcriptionally suppressed under standard laboratory culture conditions; all but seven of these are transcriptionally upregulated when the fungus is exposed to suberyylanilide hydroxamic acid or 5-azacytidine (Fisch et al., 2009). Small-molecule toxins targeting different cellular processes, cycloheximide, amphotericin B and 5-fluorocytosine, did not induce similar changes in natural product production in this organism. In a related study, the addition of suberyylanilide hydroxamic acid to semi-solid cultures of A. niger yielded a new metabolite containing a unique 1-phenylpyridin-4 (1H)-one core, nygerone A (Fig. 2) (Henrikson et al., 2009). Nygerone A was not detected when A. niger was grown under varied fermentation conditions (not reported).

Histone deacetylase inhibitors include peptides. The cyclodepsipeptide jasplakinolide elicited synthesis of three new metabolites in Phomopsis asparagi (Christian et al., 2005). Chaetoglobosin-542 and -540 (Fig. 2) exhibited cancer cell line cytotoxicity and disrupted actin filaments, while chaetoglobosin-510 (Fig. 2) was inactive in these screens. The polypeptide antibiotic goadsporin induces antibiotic activity (not isolated) in a wide variety of streptomycetes (Onaka et al., 2001). Whether jasplakinolide and goadsporin specifically inhibit histone deacetylase or have another target that results in an altered metabolome remains to be seen. One target of jasplakinolide is known; it stabilizes actin filaments in vitro, but can disrupt actin filaments in vivo.

Oligosaccharides

In Penicillium chrysogenum, mannan oligosaccharides (MO) enhance penicillin G levels and penicillin G intermediate, increase germination rates, hyphal tip numbers, clump area and spore counts, and decrease levels of reactive oxygen species (Ariyo et al., 1998; Tamerler et al., 2001; Radman et al., 2004a,b, 2006). Early evidence that increased production of penicillin in the presence of alginate or alginate-derived oligomannuronate (OM) is due to higher transcript levels of the three penicillin biosynthetic genes penCB, penC and penDE was provided by Gang and colleagues (2001), using Northern analysis and reporter gene studies. The stimulatory effect was observed in a defined medium and to a lesser extent in a complex medium containing corn steep liquor. The authors postulate that the difference could be due to the presence of elicitors in corn steep.

The absolute quantification of penicillin biosynthetic gene copy number in the presence of MO and OM was recently reported using quantitative PCR (Nair et al., 2009). Increased production was noted in both shaken flasks and bioreactors (where pH, oxygen concentration and temperature were maintained) containing P. chrysogenum. In shaken flasks, penicillin production was 133% higher in single elicitor-added cultures (MO added 48 h post-inoculation) compared with control cultures. Production increased another 71% when two different elicitors were used (MO at 48 h followed by OM at 96 h). Increased transcription levels were also similar in shaken flasks and bioreactors, with an increase of 216%, 61% and 200% in the pcbAB, pcbC and penDE transcript levels for single elicitor-added cultures over control cultures. Addition of a second elicitor resulted in increases of 116% (pcbAB), 39% (pcbC) and 33% (penDE) transcript levels compared with single elicitor cultures. Strains of P. chrysogenum synthesize the pigment chrysogenum. Alginate and pectin oligosaccharides enhance chrysogenum production up to 40% in shake flasks and bioreactors (Asilonu et al., 2000). Adding the oligosaccharide elicitors at the beginning of fermentation did not have a detectable effect on secondary metabolite production. The optimal time for elicitor addition was 24–48 h, but chrysogenum yields improved through 72 h.

Oligosaccharides also elicit increased synthesis of antibiotics in bacteria. Bacitracin yield improvements of 29%, 27% and 16% over control Bacillus licheniformis cultures were achieved in a defined medium with oligoguluronate (OG), MO and OM respectively (Murphy et al., 2007a). When OG was added at 0 h and MO at 24 h, bacitracin production was enhanced a further 13.2%, while pH profiles and biomass were unaffected (Murphy et al., 2007b). Evidence for transcriptional level control was obtained using real-time PCR, where a direct correlation between enhancement of bacitracin A production and the transcription of its biosynthetic genes was made. Elicitor supplementation (OG and MO as above) resulted in increased transcription of the bacitracin biosynthetic genes bacABC (Murphy et al., 2007b), and overexpression of bcrABC.
genes encoding the bacitracin ABC transporter system (Murphy et al., 2008). Polysaccharides have also been used to enhance production of secondary metabolites in bacteria and fungi (Patterson and Bolis, 1997; Zhu et al., 2008).

**Enzyme inhibitors**

Enzyme inhibitors can influence secondary metabolite production. Liang and colleagues (2010) tested the hypothesis that P450-type enzymes, involved in the regulation of biosynthesis of secondary metabolites such as triterpenoids, could potentially increase production of anti-tumor ganoderic acids. The addition of 100 mM phenobarbital (P450 inducer) to a two-stage cultivation involving shake culture followed by static culture resulted in an increase in the levels of ganoderic acid-Mk, -T, -S and -Me by 47%, 28%, 36% and 64% respectively. Transcription of three key genes in the triterpene biosynthetic pathway, 3-hydroxy-3-methylglutaryl coenzyme A reductase, squalene synthase and lanosterol synthase, was upregulated under phenobarbital induction.

Lycopene is a precursor of cyclic carotenoids and undergoes a number of metabolic reactions (e.g. cyclization) to form carotenoids (Bhosale, 2004). The chemical inhibitors pyridine and imidazole stimulate lycopene formation in *Blakeslea trispora* by inhibiting the enzymes responsible for cyclization of lycopene.

Two new bisnaphthalenes, sphaerolone and dihydrosphaerolone (Fig. 2), from the accumulated 1,3,8-trihydroxynaphthalene, were isolated when *Sphaeropsis* sp. was grown in the presence of enzyme inhibitors such as tricyclazole (Bode and Zeeck, 2000), an antifungal that inhibits 1,3,8-trihydroxynaphthalene reductase. A new macrolide mutolide (Fig. 2) was also elicited by tricyclazole in this strain (Bode et al., 2000a). Mutolide had weak antibacterial activity. Interestingly, both UV mutagenesis and tricyclazole apparently activate a silent gene cluster responsible for mutolide biosynthesis (Bode et al., 2000a).

**Solvents and heavy metals**

Organic compounds such as ethanol or dimethylsulfoxide (DMSO) have also been used to elicit secondary metabolite biosynthesis. Ethanol and DMSO cause mistranslation (So and Davie, 1964, 1965), and presumably have other mechanisms such as induction of the stress response (Chen et al., 2000). Although their precise mechanism (more likely multiple mechanisms) of inducing secondary metabolite synthesis is unknown, they are included due to their ease of use, inexpensive nature and dramatic results.

In the presence of 3% DMSO, chloramphenicol and tetracenomycin C production increased approximately threefold in *Streptomyces venezuelae* and *S. glaucescens* respectively (Chen et al., 2000). Thiostrepton production increased about twofold in *S. azureus* with 3% DMSO. When treated and untreated cultures were compared, no significant differences in biomass were seen. These elicitation results are remarkable given that these antibiotics are from three different biosynthetic families. Substantial differences in HPLC patterns and increased pigment production were noted when cultures of three other species of *Streptomyces* were supplemented with DMSO. Dimethyl sulfone, a metabolite of DMSO, had similar but less pronounced stimulatory effects on antibiotic production. Ethanol (1–3%) stimulated tetracenomycin C production, but inhibited chloramphenicol production.

Ethanol (1%) elicited synthesis of a new chlorinated benzophenone antibiotic, pestalone, by the marine fungus *Pestalotia* (Cueto et al., 2001) (Fig. 2). Pestalone has potent activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium*, and marginal activity against the National Cancer Institute’s 60 human tumour cell line screen. The antibiotic is undetectable in non-elicited *Pestalotia* cultures. Pestalone is also synthesized when *Pestalotia* is co-cultured with a marine bacterium (Cueto et al., 2001).

Ethanol (6%) dramatically increases synthesis of the benzoazolophenanthridine antibiotic jadomycin B in *S. venezuelae* (Doull et al., 1994). Addition of ethanol (0.2%) to cultures of *Phaffia rhodozyma* resulted in increased carotenoid production, apparently due to activation of oxidative metabolism and induction of HMG-CoA reductase (Gu et al., 1997). Polyphenol production (and biomass) by the medicinal fungus *Inonotus obliquus* is enhanced by the continuous addition of a low concentration of hydrogen peroxide (1 mM) to batch cultures (Zheng et al., 2009). Superoxide dismutase and catalase activities were also enhanced.

In a review of the effect of heavy metals on secondary metabolite production/cellular differentiation, Weinberg (1990) summarizes evidence that regulation primarily occurs at the level of transcription. Furthermore, three heavy metals, Mn2+, Fe3+ and Zn2+, are identified as having a key role in the secondary metabolism of a wide variety of microorganisms. In a more recent study, non-toxic concentrations of the heavy metal ions Cu2+, Cd2+ and Cr3+ significantly increased production of the polyketide monocolin I by the plant-associated fungus *P. quadireptata* (Paranagama et al., 2007). Extracts of metal tolerant actinobacteria grown in the presence of Ni2+ (in either complex or minimal media) inhibited the growth of a variety of bacteria and fungi (Haferburg et al., 2009).

Because many antibiotics and other secondary metabolites can scavenge heavy metals (Demain and Fang, © 2010 The Author

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2000), the authors propose that synthesis of such antimicrobial metabolites is increased when metals are added to the fermentation medium of heavy metal tolerant strains (Haferburg et al., 2009). Certain enzymes require metals as cofactors. Mn$^{2+}$, for example, is a cofactor for enzymes involved in carotenoid synthesis, and this heavy metal has been shown to enhance carotenoid production in various microbes (Bhosale, 2004). Other heavy metal ions, La$^{3+}$, Ce$^{3+}$ and Nd$^{3+}$, had a stimulatory effect on carotenoid synthesis in *Phaffia rhodozyma*, while slightly inhibiting growth (Wang et al., 1999).

Conclusions and prospects for the future

Results of genome analyses reveal why manipulation of microbial growth can be a fertile avenue for secondary metabolite discovery. Certain groups of bacteria and fungi have dozens of secondary metabolite pathways that are not expressed under standard laboratory growth conditions. Microbial small-molecule secondary metabolites resulting from several billion years of biosynthetic reactions have been a tremendous resource for natural product drug discovery, and their usefulness will continue if the substantial proportion of cryptic biosynthetic pathways can be accessed.

Transcriptional suppression of secondary metabolites may be a mechanism to protect microbes from autotoxicity. Biosynthesis of metabolically expensive and potentially autotoxic secondary metabolites is presumably only elicited when an appropriate environmental trigger is encountered (Patterson and Bolis, 1997; Fisch et al., 2009). The use of small-molecule elicitors, in some cases targeted, non-random elicitors such as methyltransferase and histone deacetylase inhibitors, allows researchers to overcome transcriptional suppression of potentially important gene clusters in the laboratory. To date, more than a dozen new compounds from microbes have been identified with small-molecule elicitation. Enhanced production of rare metabolites is another important application of this method, and there are numerous reports of increased production of well-known metabolites using elicitors. Interestingly, the *de novo* production of some secondary metabolites can be elicited with both a small molecule or UV (mutolide), or a small molecule or co-culture (pestalone).

Treatment of microbes with one of the many small-molecule elicitors is a simple, inexpensive method to more thoroughly exploit the metabolic potential of microbes. Small-molecule elicitation allows identification of new secondary metabolites and enhanced synthesis of low yield secondary metabolites in a broad spectrum of microorganisms. These types of experiments can be performed in almost any lab, and are much less cumbersome than varying myriad cultivation parameters. Furthermore, this technique uses native hosts as opposed to heterologous hosts, which can be fraught with complications.

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