microbial biotechnology

Microbial Biotechnology (2011) **4**(4), 471–478 doi:10.1111/j.1751-7915.2010.00196.x

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, nygerone 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.

Aspergilli 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-*

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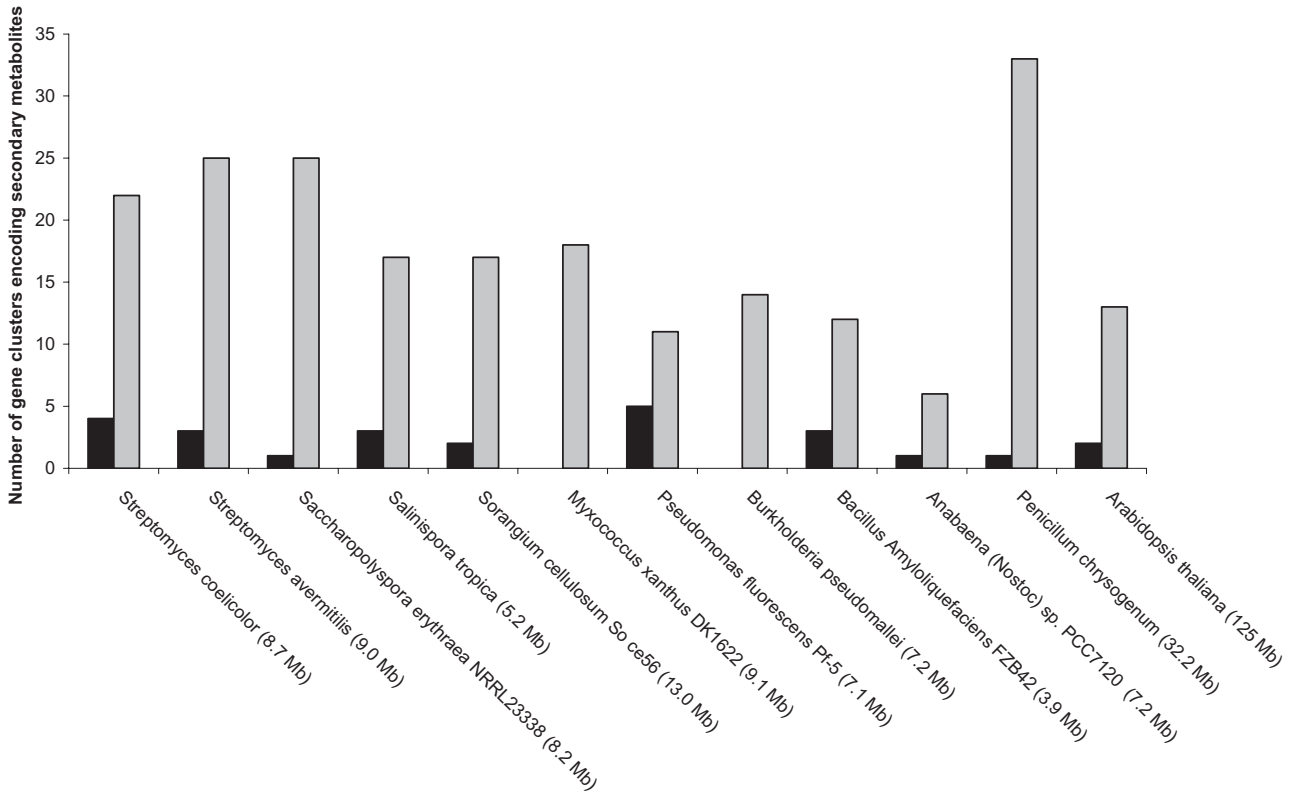

Fig. 1. Selected fully sequenced prokaryotic and eukaryotic secondary metabolite producers (Gross, 2009). Dark bars show the numbers of gene clusters known to be present in the genome, based on the number of isolated secondary metabolites at the time of sequencing. Light bars show the total number of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

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 *Sphaeropsidales* sp., which synthesizes the antifungal spirobisnaphthalene cladospirone 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 *Paraphaeosphaeria quadriseptata*, cytosporones F-I, quadriseptin A and 5′-hydroxymonocillin III (Paranagama *et al*., 2007). When grown in a modified malt extract medium containing soluble starch instead of glucose, *Gymnascella dankaliensis* 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.

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 CclA (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, ^{1}H-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-

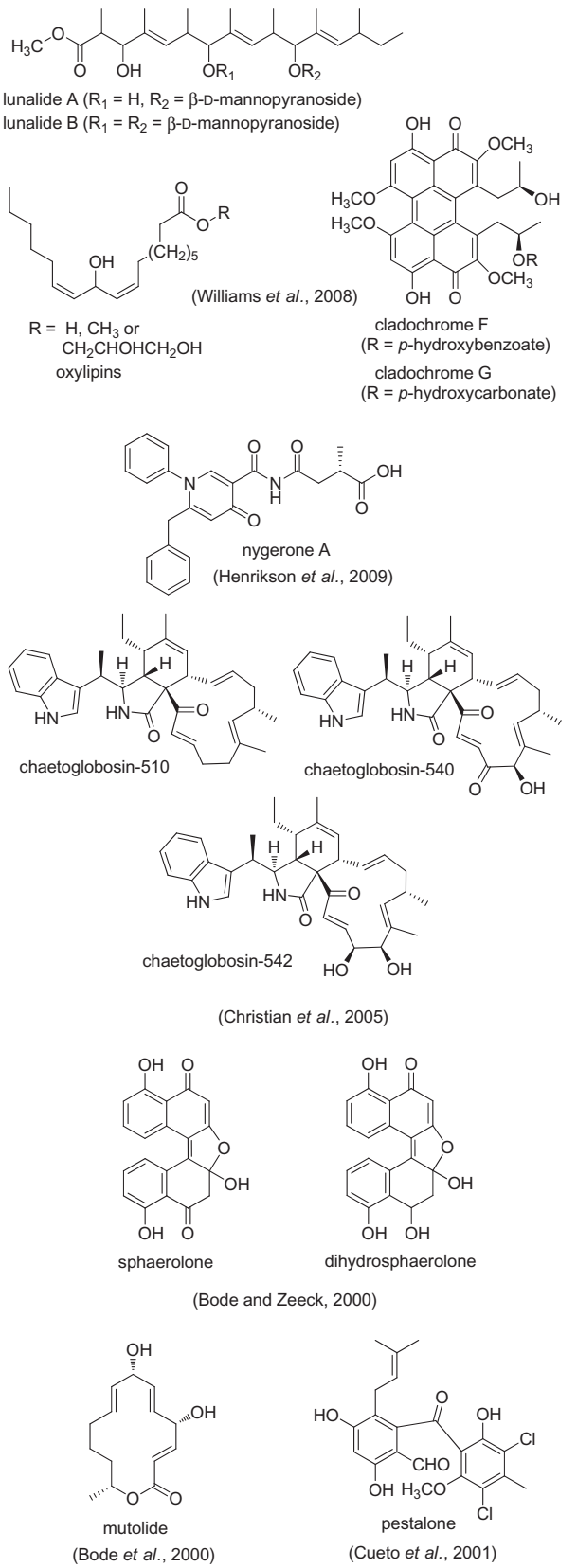

Fig. 2. Novel microbial secondary metabolites elicited with small molecules.

eroylanilide hydroxamic acid led to dramatic restructuring of its metabolome (Williams *et al*., 2008). 5-azacytidine elicited the *de novo* production of several oxylipins in *C. cladosporioides* (Fig. 2). Suberoylanilide hydroxyamic 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* 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 suberoylanilide 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 suberoylanilide 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 secondary metabolites in *Phomospis 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 intermediates, 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 *pcbAB*, *pcbC* 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 chrysogenin. Alginate and pectin oligosaccharides enhance chrysogenin 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 chrysogenin 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 μM 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 *Sphaeropsidales* 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 benzoxazolophenanthridine 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, Mn^{2+}, Fe^{3+} and Zn^{2+}, 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 Cu^{2+}, Cd^{2+} and Cr^{3+} significantly increased production of the polyketide monocillin I by the plant-associated fungus *P. quadriseptata* (Paranagama *et al*., 2007). Extracts of metal tolerant actinobacteria grown in the presence of Ni^{2+} (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,

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 such 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.

Acknowledgements

Financial support was provided by grants R01 CA90441-01-05, 2R56 CA090441-06A1, and 5R01 CA090441-07 from the Division of Cancer Treatment, Diagnosis and Centers, National Cancer Institute, DHHS, and the Arizona Biomedical Research Commission. Many thanks to F. Hogan for preparing Figure 2.

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