FEMS Microbiology Reviews, fuw035, 41, 2017, 19–33

doi: 10.1093/femsre/fuw035
Advance Access Publication Date: 29 August 2016
Review Article

REVIEW ARTICLE

Antibiotic dialogues: induction of silent biosynthetic gene clusters by exogenous small molecules

Bethany K. Okada[1] and Mohammad R. Seyedsayamdost[1,2,*]

[1]Department of Chemistry, Princeton University, Princeton, NJ 08544, USA and [2]Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA

*Corresponding author: Departments of Chemistry and Molecular Biology, Princeton University, Princeton, NJ 08544, USA. Tel: 609-258-5941; Fax: 609-258-8163; E-mail: mrseyed@princeton.edu

One sentence summary: Microbial secondary metabolites represent a significant source of potential drug leads; however, the majority of the corresponding biosynthetic genes are not expressed under normal laboratory conditions. In this review, we assess the capacity of exogenous small molecules, especially antibiotics, to activate these silent gene clusters.

Editor: Aimee Shen

ABSTRACT

Natural products have traditionally served as a dominant source of therapeutic agents. They are produced by dedicated biosynthetic gene clusters that assemble complex, bioactive molecules from simple precursors. Recent genome sequencing efforts coupled with advances in bioinformatics indicate that the majority of biosynthetic gene clusters are not expressed under normal laboratory conditions. Termed 'silent' or 'cryptic', these gene clusters represent a treasure trove for discovery of novel small molecules, their regulatory circuits and their biosynthetic pathways. In this review, we assess the capacity of exogenous small molecules in activating silent secondary metabolite gene clusters. Several approaches that have been developed are presented, including coculture techniques, ribosome engineering, chromatin remodeling and high-throughput elicitor screens. The rationale, applications and mechanisms attendant to each are discussed. Some general conclusions can be drawn from our analysis: exogenous small molecules comprise a productive avenue for the discovery of cryptic metabolites. Specifically, growth-inhibitory molecules, in some cases clinically used antibiotics, serve as effective inducers of silent biosynthetic gene clusters, suggesting that old antibiotics may be used to find new ones. The involvement of natural antibiotics in modulating secondary metabolism at subinhibitory concentrations suggests that they represent part of the microbial vocabulary through which inter- and intraspecies interactions are mediated.

Keywords: secondary metabolites; antibiotics; interspecies interaction; ribosome engineering; chromatin remodeling; HiTES

INTRODUCTION

Natural products have had a profound impact on the discovery of new therapeutic agents. Newman and Cragg have highlighted their critical roles in modern medicine, and in a series of reports, compiled comprehensive data sets for all major groups of drugs (Newman and Cragg 2009, 2016; Cragg and Newman 2013). Accordingly, in the period between 1981 and the end of 2014, 60% of all small-molecule drugs approved were derived from natural products. These molecules have been especially effective as antibiotics and anticancer agents. Of the 175 small molecule anticancer agents approved since the 1940s, 49% are either natural products or directly derived thereof. A staggering 73% of small molecule antibiotics are natural products or their derivatives. Soil-dwelling actinomycetes have been major contributors in this regard as over 50% of clinical antibiotics are produced by this group of prolific bacteria (Berdy 2005).

Most antibiotic scaffolds in use today were discovered between 1940 and the early 1960s, a period now referred to as the golden age of antibiotics discovery. This productive era was

Received: 3 June 2016; **Accepted:** 29 July 2016

followed by a ~40 year innovation gap, during which no new antibiotic scaffolds were introduced (Walsh and Clatworthy, Pierson and Hung 2007; Fischbach and Walsh 2009). The consequences of this gap have been dire, especially considering the emergence of multidrug-resistant bacterial pathogens such as methicillin-resistant *Staphylococcus aureus*, extensively drug-resistant *Mycobacterium tuberculosis* or pan-drug-resistant *Pseudomonas aeruginosa*, to name a few. Resistance has been detected to every antibiotic that is currently on the market, and this observation coupled with our inability to identify new effective antibiotics has catapulted infectious diseases to one of the main health challenges of this century. The Center for Disease Control and Prevention recently reported that annually over 2 million Americans are infected by drug-resistant pathogens; over 23 000 of these are fatal. If new methods for discovery of bioactive molecules are not developed, infectious disease may again become the leading cause of death worldwide.

Traditional methods, now often referred to as 'grind and find', were for decades an excellent source of diverse, potent and inspirational new molecules (Miller and Clardy 2009). However, their application often leads to labor-intensive dereplication of previously known compounds. Due to diminishing returns and the relatively limited profit margin of antibiotics, major pharmaceutical companies have for the most part abandoned antibiotics discovery pipelines (Projan 2003). This decision might have been premature, not only because restocking our antibiotic supplies is a public health priority, but also because modern advances in genome sequencing technologies and the development of bioinformatics have reinvigorated antibiotic discovery. The abundance of bacterial genome sequences now available enables genome-mining approaches for finding new bioactive small molecules, while simultaneously facilitating cheminformatic dereplication methods (Gaudêncio and Pereira 2015; Mohamed, Nguyen and Mamitsuka 2016).

Microbial natural products are generated by dedicated biosynthetic gene clusters. The corresponding biosynthetic enzymes assemble, in a stepwise fashion, architecturally complex secondary metabolites from simple building blocks. One of the main insights from the multitude of microbial genome sequences has been that most secondary metabolite biosynthetic gene clusters are inactive during normal laboratory fermentation (Bentley *et al*. 2002; Ikeda *et al*. 2003; Oliynyk *et al*. 2007; Nett, Ikeda and Moore 2009). *Saccharopolyspora erythraea*, the industrial producer of erythromycin, provides an illustrative case. While its genome revealed 27 biosynthetic clusters, decades of research have only uncovered products for five of these. The remaining majority, termed 'silent' or 'cryptic' gene clusters, represent a treasure trove for the discovery of novel small molecules, their biosynthetic pathways and the regulatory circuits underlying their expression. These data lead to two profound conclusions: (1) our current arsenal of naturally derived drugs has been acquired from a small fraction of constitutively expressed biosynthetic gene clusters and (2) methods that access the silent majority would have a deep impact on drug discovery and increase our collection of bioactive molecules.

The research community has recognized that silent gene clusters represent a large reservoir of therapeutic molecules, and several methods have been developed for activating them (Chiang *et al*. 2011; Rutledge and Challis 2015). These include expression of the entire gene cluster in a suitable heterologous host, chromosomal insertion of constitutively active promoters, overexpression of pathway-specific regulators and the OSMAC (One Strain Many Compounds) approach (Bode *et al*. 2002). Herein, we consider the use of exogenous small molecules in modulating the expression of silent gene clusters. We present the major approaches that have been developed—including coculture techniques, ribosome engineering, chromatin remodeling and high-throughput elicitor screens—and discuss each in terms of rationale, applications and mechanism. We exclude quorum-sensing-regulated pathways from our analysis, as the autoinducers are known and largely produced by the host, and consequently the corresponding gene clusters are not silent. We also exclude OSMAC from our discussion. While successful, it is in most cases unclear what media component(s) induce expression of silent gene clusters. Our analysis underlines the power of exogenous small molecules in stimulating secondary metabolism and highlights the function of elicitors, especially antibiotics, in mediating microbial interactions and inducing the production of cryptic secondary metabolites.

COCULTURE

Motivation

Traditional bacterial natural products discovery often involves growth of a selected strain in a nutrient-rich monoculture. Though effective, this culturing method is in stark contrast to the complex, nutrient-limited environment in which bacteria naturally grow and evolve. One gram of soil, for example, contains 10 000 different species of bacteria (Curtis, Sloan and Scannell 2002). As such, inter- and intraspecies interactions are prevalent in the wild, and it is perhaps unsurprising that growth of a bacterium in coculture with a potential competitor would alter or enhance the secondary metabolic output. It would stand to reason then that systematic coculture screens could lead to new, perhaps cryptic, bioactive molecules that cannot be accessed otherwise. Early support for this idea came from Peipp and Sokolova (Iakovleva and Sokolova 1978; Sonnenbichler, Dietrich and Peipp 1994). Peipp, for example, found that constitutive, albeit low-level, production of a toxin by the fungus *Heterobasidion annosum* stimulated the biosynthesis of several toxins by a second fungus, *Gloeophyllum abietinum*, in liquid coculture. These overproduced toxins included oosponol (~92-fold) and oospoglycol (~14-fold) (Sonnenbichler, Dietrich and Peipp 1994). While numerous applications have followed, this method largely remains an underexplored strategy for the discovery of new and cryptic small molecules (Kolter and van Wezel 2016).

Applications

Relevant applications of coculture may be traced back to an entirely different area: foods and beverages such as vinegar, beer, chocolate and cheese have long been produced in cocultures, which take advantage of interactions between multiple microorganisms. The first example of coculture in natural products discovery, though also 'unintended', is that of penicillin, produced by *Penicillium notanum* on a petri dish containing *Staphylococcus aureus*. The observation of a halo of staphylococcal growth inhibition by Alexander Fleming and further developments by Walter Florey and Ernst Chain revolutionized the treatment of bacterial infections. However, neither of these examples represents a methodical microbial coculture experiment. Testing of bacterial and fungal extracts against other bacteria continued for the following decades, but systematic coculture studies only became popular toward the end of the century (Iakovleva and Sokolova 1978; Sonnenbichler, Dietrich and Peipp 1994; Mearns-Spragg *et al*. 1998; Ueda *et al*. 2000). Broadly speaking, induction of silent

gene clusters as a function of coculture can result from one of three interaction modes: (1) provision of nutrients, (2) competition via production of an antibiotic or a signaling molecule and (3) cell–cell contact. Examples in each of these categories will be discussed in turn.

Application: provision of nutrients

Many of the earliest coculture experiments monitored readily apparent phenotypic changes, such as pigment production, sporulation or antibiotic synthesis. In numerous cases, diffusible small molecules from one strain elicit production of secondary metabolites from a second strain. These diffusible molecules act as either nutrients, signals or antibiotics. Examples of the first category were presented by Ueda and coworkers in a broad binary screen with 76 strains of *Streptomyces*. In one set of experiments, they found that 34% of the Streptomycetes tested were active in inducing antibiotic production and/or sporulation in neighboring Streptomycetes (Ueda *et al*. 2000). These results indicated that *Streptomyces* spp. harbored cryptic antibiotics well before the genome sequence of the first actinomycete was reported. Additional studies with one of the interacting pairs identified the siderophore desferrioxamine E (Fig. 1, **1**) as the stimulatory molecule (Yamanaka *et al*. 2005). Other siderophores did not display this phenotype. These results indicated that iron, delivered by specific siderophores, plays an important role in Streptomycete development. Consistent with this model, siderophores that monopolize iron rather than provide it to the neighboring strain retard developmental pathways, as shown in the case of *Amycolatopsis* sp. AA4 and *Streptomyces coelicolor* (Seyedsayamdost *et al*. 2011b; Traxler *et al*. 2012).

An early intrakingdom case in this category involved a binary fungal interaction with immediate industrial relevance: coculture of *Rhodotorula glutinis* and *Debaryomyces castellii* resulted in increased carotenoid pigment production that was not seen in either monoculture (Buzzini 2001). The authors suspected that *D. castellii* hydrolyzes the oligosaccharides in the medium to mono- and disaccharides, which *R. glutinis* can use more effectively in carotenoid synthesis.

Application: competition and signaling

While nutrient provision, in the form of iron or sugar, affords one means of interaction, competition and antibiotic production itself can underlie other bacterial or fungal interactions. Many examples fall into this category. An early bacterial–bacterial example was the induction of the blue *S. lividans* pigment, actinorhodin (Fig. 1, **2**) by another *Streptomyces* strain, found by screening supernatants from 405 strains of actinomycetes (Onaka *et al*. 2001). The signaling compound was isolated and identified as a thiazole/oxazole-containing linear oligopeptide, which the authors named goadsporin (Fig. 1, **3**). Goadsporin proved to be a more general inducer of Streptomycete secondary metabolism: it was tested against 42 randomly selected strains and elicited pigment production in 20 strains and sporulation in 32 strains. Interestingly, goadsporin was found to be a potent *Streptomyces*-specific antibiotic: it did not show bioactivity against selected proteobacteria, firmicutes or fungi, but exhibited minimal inhibitory concentrations of 0.2, 3.2 and 6.4 μg mL^{-1} against *S. scabies*, *S. coelicolor and S. lividans*, respectively. At subinhibitory concentrations, goadsporin stimulated sporulation and antibiotic production, while at high concentrations, it served as a potent toxin. This phenomenon of low-dose stimulation and high-dose toxicity by the same molecule is referred to as hormesis, and the following sections will show that it is a prevalent interaction paradigm between microbes and molecules.

A case similar to goadsporin was provided by promomycin (Fig. 1, **4**). It was discovered as an elicitor in an interaction where one *Streptomyces* strain promoted production of an otherwise cryptic antibiotic in another (Amano *et al*. 2010). The eliciting molecule, promomycin, was shown to be a polyether antibiotic, structurally similar to lonomycin. Like goadsporin, promomycin displayed hormetic properties. At low concentrations, it served a stimulatory role for antibiotic production, while at high concentrations, it displayed antibacterial properties (Fig. 2A). The similarity to polyether antibiotics led the authors to use monensin A (Fig. 1, **5**) as an elicitor, which eventually resulted in the identification of the antibiotic elicited from the second strain (Amano *et al*. 2011). Although the level of induction appeared weak, the authors elucidated its structure as the diisonitrile-bearing antibiotic, SF2768 (Fig. 1, **6**).

Of note in the category of toxin-inducing-toxin cocultures are also results by Shin *et al*. (1998), which showed that a fungal *Monascus* species, grown with either *Saccharomyces cerevisiae* or *Aspergillus oryzae*, exhibited ~11-fold enhanced pigment production and morphological differentiation. Remarkably, the inducer provided by *Sa. cerevisiae* was shown to be a chitinase enzyme. Among a number of degradases tested, the *Sa. cerevisiae* chitinase was shown to be the most effective at hydrolyzing *Monascus* cell walls. The authors suggested that partial hydrolysis of *Monascus* cell walls served as a signal for stimulation of pigment production, which they proposed was a form of chemical defense.

A similar example involving a fungal–bacterial interaction was reported by Patterson and Bolis. They showed that fungal cell wall homogenates from *P. notatum* elicited a 2.5-fold upregulation of tolytoxin by the cyanobacterium *Scytonema ocellatum* (Patterson and Bolis 1997). Further, they identified the inducer as variable oligomers of *N*-acetylglucosamine, that is, chitin polymers of differing lengths. Interestingly, the inducer again displayed hormetic properties: high concentrations of the homogenate and of chitin oligomers were toxic, while low concentrations led to tolytoxin overproduction. The authors concluded that tolytoxin is an inducible chemical defense agent, known as a phytoalexin. Much like Streptomycetes, this cyanobacterium also responds to *N*-acetylglucosamine by turning on toxin production pathways (Rigali *et al*. 2008). Similar interactions have been shown in marine bacterial–bacterial interactions as well as algal–bacterial interactions (Long and Azam 2001; Seyedsayamdost *et al*. 2011a).

Examples where the elicitors are not growth inhibitory have also been reported. Akin to the studies of Patterson and Bolis above, stimulation of *Streptomyces* sp. US80 with heat-killed fungi led to a modest overproduction of antifungal metabolites, including the potent toxin irumamycin (Fourati-Ben Fguira *et al*. 2008). In this case, no growth inhibition of the bacterial strain was reported. Further, Proksch and colleagues have reported a 1.5- to 78-fold overproduction of bioactive metabolites from the ascomycete *Fusarium tricinctum* when cocultured with *Bacillus subtilis* (Ola *et al*. 2013). These studies led to identification of four cryptic metabolites, three of which are known to be produced by the fungus. Interestingly, these results depended on the duration of pre-incubation of *B. subtilis* on agar media before introduction of the fungus. Best results were obtained with a 6-day pre-incubation of *B. subtilis*, perhaps suggesting a growth phase-dependent production of a signal by the bacterium.

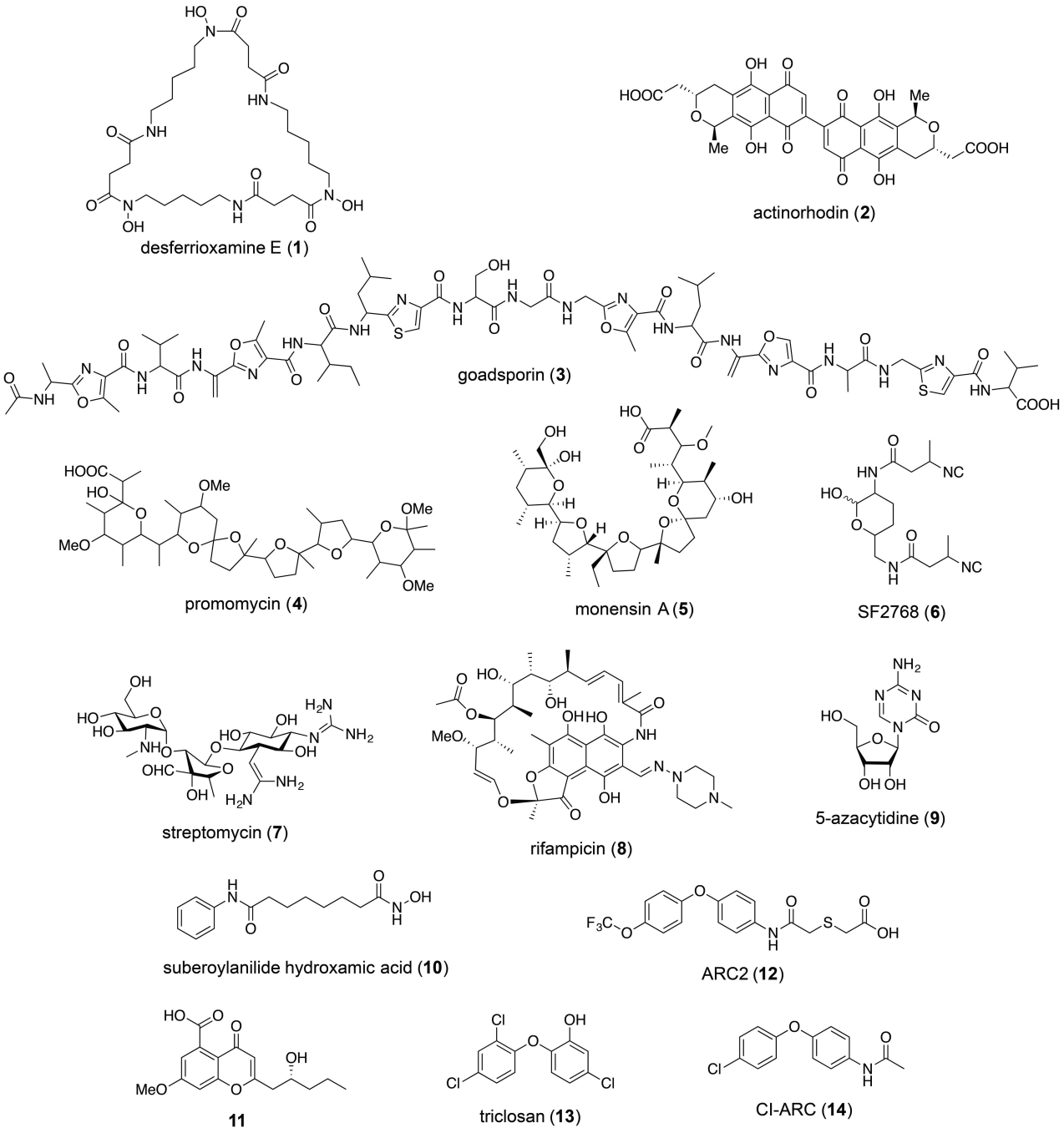

Figure 1. Selected structures, including desferrioxamine E (1), actinorhodin (2), goadsporin (3), promomycin (4), monensin A (5), SF2768 (6), streptomycin (7), rifampicin (8), 5-AC (9), SAHA (10), bortezomib-induced metabolite (11), ARC2 (12), triclosan (13) and Cl-ARC (14).

As was alluded to above, a study in the bacterial–bacterial interactions category showed that exposing unidentified, surface-associated marine bacteria to *St. aureus*, *Pseudomonas aeruginosa* or *Escherichia coli*, resulted in increased antibiotic synthesis by the marine bacteria. This study, much like those by Ueda, reported the production of cryptic antibiotics from marine bacteria and indicated that the observed metabolic changes depended on an unidentified signal (Mearns-Spragg *et al.* 1998). In a similar approach, Slattery, Rajbhandari and Wesson (2001) examined the effect of 53 diverse bacterial species in coculture with the marine istamycin producer, *Streptomyces tenjimariensis*. They reported that 23% of interactions tested led to a ~2-fold overproduction of istamycin. Their results were interpreted as an inducible defense mechanism in the marine microbe elicited by Gram-positive and Gram-negative bacteria. Despite the low level of overproduction, these studies were fully in line with an altered metabolism in response to coculture.

Application: cell–cell contact

The last category in the mode of coculture interactions mentioned above is physical cell–cell contact. Perhaps the first

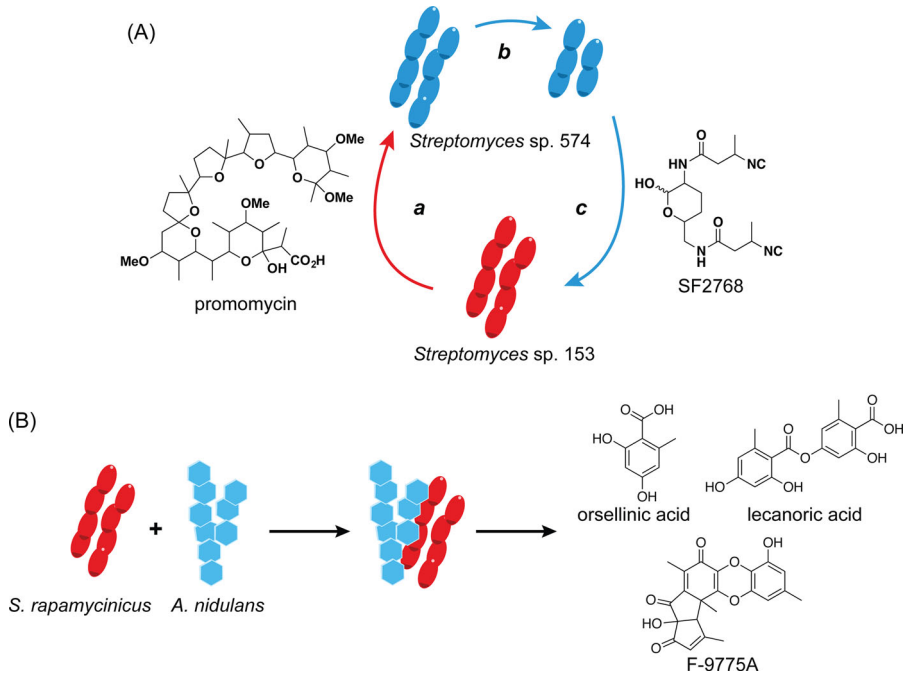

Figure 2. Stimulation of secondary metabolism by coculture techniques. (**A**) Promomycin, a polyether antibiotic, is released by one *Streptomyces* strain (***a***), which causes growth inhibition (***b***) and production of SF2768, a diisonitrile antibiotic (**c**), from a second Streptomycete. (**B**) A physical interaction between *S. rapamycinicus* and *A. nidulans* induces the fungus to produce orsellinic acid, lecanoric acid and F-9775A.

example in this category was demonstrated by Clardy and colleagues in the induction of a cryptic metabolite through coculture of a marine fungus with an unidentified Gram-negative marine bacterium (Cueto *et al.* 2001). These studies led to isolation and structural elucidation of pestalone, a benzophenone antibiotic, and demonstrated that it was not detectable in discrete fungal and bacterial controls. Pestalone displayed potent antibacterial activities with MICs of 37 and 78 ng mL^{-1} against MRSA and VRE, respectively. A similar study from the Fenical lab showed that *Libertella*, a marine fungus, could be induced by a marine proteobacterium to produce the otherwise cryptic libertellenones (Oh *et al.* 2005). This new series of pimarane diterpenoids demonstrated moderate to potent cytotoxicity against a cancer cell line. In both of these studies, attempts to replicate the interaction by substituting dead bacterial cells, cell-free supernatant or ethyl acetate extracts failed, consistent with a requirement for contact in the induction of pestalone and libertellenone biosyntheses.

A more recent study on a bacterial–fungal interaction utilized a clever secondary metabolite gene microarray of *A. nidulans* in coculture with 58 soil-dwelling actinomycetes to assess activation of silent fungal biosynthetic gene clusters. The studies revealed that coculture with *S. rapamycinicus* induced production of the cryptic metabolite orsellinic acid and its derivatives (Schroeckh *et al.* 2009). Additional experiments addressing the mode of induction showed that orsellinic acid was not produced when the two species were separated by a dialysis tube or even when the bacteria were grown with a knockout strain of *A. nidulans*, and the resulting supernatant applied to the wt fungus. These experiments indicated that a diffusible signal was not involved. When the authors switched tactics and examined scanning electron micrographs of coculture biomass, they saw that the bacteria nested inside of the fungus, anchoring themselves to the fungal hyphae. Thus, in these cases, the physical interaction between the microorganisms was responsible for their altered metabolic output (Fig. 2B). For many more examples of mixed fermentation, we refer the reader to two comprehensive reviews by Pettit and Proksch (Pettit 2009; Marmann *et al.* 2014).

Mechanism

Mechanistic considerations for the various vignettes presented above will be discussed in regard to the mode of interaction, though it should be mentioned at the outset that relatively few cocultures have been studied in enough detail to implicate a specific regulatory induction pathway. The stimulatory role in antibiotic production and sporulation ascribed to desferrioxamine E (Fig. 1, **1**) can be rationalized in light of the crucial requirement of iron in Streptomycete development. Indeed, studies by Traxler *et al.* (2012) have demonstrated that expression of genes involved in the developmental program of *S. coelicolor* are strongly altered in response to changes in iron availability: abundance of iron leads to accelerated developmental pathways, while iron sequestration or limitation slows them down. Complete removal of iron causes growth retardation and failure to produce aerial hyphae. As desferrioxamine is one of the most common siderophores among terrestrial actinomycetes, the results by Ueda and colleagues indicated that provision of this siderophore by one strain supplemented the amount of iron available to the receiving strain in the binary interaction, thus promoting development including sporulation and secondary metabolite production (Yamanaka *et al.* 2005). This conclusion is consistent with the arrested development that occurs when *S. coelicolor* is provided with a rare siderophore that it cannot utilize.

Many cocultures involve a growth-inhibitory molecule produced by one microbe, which stimulates secondary metabolite production, often antibiotics, from the neighboring microbe.

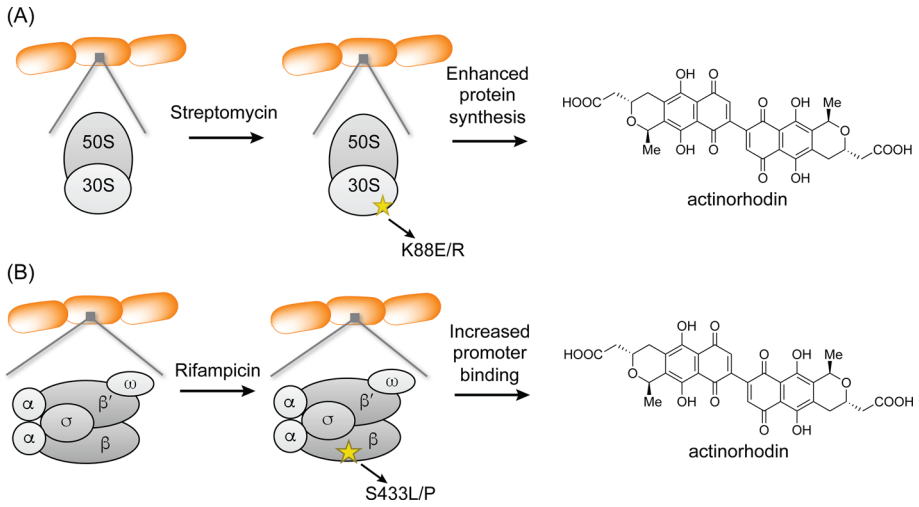

Figure 3. Stimulation of secondary metabolism by ribosome engineering. (**A**) Streptomycin treatment produces a resistant strain with a mutation in the S12 protein, a component of the 30S subunit of the ribosome. The mutated ribosome endows the streptomycin-resistant strain with enhanced protein synthesis, resulting in increased actinorhodin production. (**B**) Rifampicin treatment produces a resistant strain with a mutation in the β-subunit of RNAP. The mutated protein exhibits enhanced promoter binding, resulting in increased actinorhodin production.

These cases quite explicitly demonstrate the hormetic properties of antibiotics. At a subinhibitory concentration, the antibiotic acts as an elicitor or inducer of silent biosynthetic gene clusters resulting in production of cryptic metabolites. At higher concentrations, that same antibiotic has toxic effects on the receiving strain. These experiments seem to indicate that in some interactions, bacteria respond to exogenous antibiotics with antibiotics. Undoubtedly, in some cases, where the elicitor was not an antibiotic, nutrient limitation may be the trigger for induced secondary metabolism. Both conditions, diminished growth from exposure to antibiotics or from nutrient limitation, elicit the same response of sporulation and secondary metabolite biosynthesis.

In the case of promomycin, which is presumably produced constitutively, the mode of induction was examined further. A key observation was provided by studies of Suh and colleagues, which showed a strong inverse relationship between secondary metabolite production and intracellular ATP levels in *Streptomyces* spp. (Meng *et al.* 2011). We posit that because promomycin and monensin are ionophores, which abolish the proton motive force that is utilized to synthesize ATP, they elicit secondary metabolite production by diminishing levels of intracellular ATP. Further studies will certainly test this hypothesis and enhance our understanding of regulatory pathways that activate secondary metabolite biosynthesis.

A number of fungal–bacterial interactions have been found to be mediated not by diffusible small molecules but rather by physical cell-to-cell contact. Brakhage and colleagues showed that interactions of *A. nidulans* and *S. rapamycinicus* fell into this category, and scanning electron micrographs beautifully visualized the process. (Schroeckh *et al.* 2009). Strikingly, when this mode of induction was examined in more detail, it was revealed that the bacteria were causing alterations to fungal chromatin by histone acetylation via the Saga/Ada complex, which contains the histone acetyl transferase GcnE (Nützmann *et al.* 2011). This provided the first example of Saga/Ada-mediated histone acetylation triggered by a bacterial interaction. As will be discussed more fully in the discussion on chromatin remodeling, fungal secondary metabolism can be highly sensitive to histone modifications.

RIBOSOME ENGINEERING

Motivation

Ribosome engineering approaches the problem of silent gene clusters from a unique angle: How can the producers themselves be improved? While numerous methods for strain improvement exist, they are typically hampered by high costs and/or laborious procedures (Santos and Stephanopoulos 2008). In contrast, ribosome engineering has emerged as a simple and rapid secondary metabolism-focused alternative. This technique is predicated on the use of certain antibiotics to produce mutations in component(s) of the ribosome or RNA polymerase (RNAP) that result in increased production of secondary metabolites. The inventors of the method were inspired by the observation that a mutated, streptomycin-resistant strain of *Streptomyces lividans* produced the blue pigment actinorhodin (Act, Fig. 1, **2**), whereas the parent strain, from which the mutant was derived, did not (Shima *et al.* 1996). They mapped the mutation to a ribosomal protein and showed that only the mutant ribosome led to activation of the silent Act gene cluster (*act*), thus demonstrating translational control in Act biosynthesis. The method has been extended to other antibiotics and many successful applications have followed (Ochi 2007; Ochi and Hosaka 2013).

Applications

Ochi and Hosaka (2013) have recently provided a comprehensive summary of the applications of ribosome engineering. Here we focus on the original studies that provided the impetus for the field as well as a molecular rationale for ribosome engineering. As alluded to above, wt *S. lividans* TK21 normally does not produce Act, even though it contains a complete *act* biosynthetic gene cluster (Shima *et al.* 1996). The original study by Ochi and colleagues investigated how the streptomycin-resistant *S. lividans* strain TK24 produced abundant quantities of Act (Fig. 3A). Resistance to streptomycin (Fig. 1, **7**) frequently results from point mutations in *rpsL*, which encodes the S12 ribosomal protein. When *rpsL* from strains TK21 and TK24 was sequenced, Shima *et al.* found a K88E mutation in TK24. Selection

of additional streptomycin mutants from TK21 showed that Act production often accompanied resistance. Convincingly, introduction of the K88E-S12 into the parent strain gave the same result, indicating that the K88E mutation was linked with the ability to induce the *act* gene cluster. These results established that activation of *act* in *S. lividans* can occur at the translational level and is dependent on a mutated or 'engineered' ribosome.

The correlation between streptomycin resistance, which arises through a specific mutation in S12, and the induction of a biosynthetic gene cluster has been extended to additional Streptomycetes, other bacterial genera and even fungi. Studies have shown that selection of streptomycin resistance in *S. coelicolor*, *S. antibioticus*, *S. chattanoogensis* and *S. lavendulae* led to a 5- to 6-fold overproduction of Act, actinomycin, fredericamycin and formycin, respectively (Hosoya *et al.* 1998). Notably, the mutants resulted not in global overproduction of secondary metabolites but rather in pathway-specific stimulatory effects (see below). Further studies have helped extend this method to *Bacillus subtilis*, *B. cereus* and *Pseudomonas pyrrocinia*, where selection of streptomycin-resistant mutants (*str*) resulted in a 5- to 10-fold overproduction of a peptidic antibiotic, FR900493, and pyrrolnitrin, respectively.

Ochi and coworkers have expanded the streptomycin-induced ribosome remodeling paradigm to other antibiotics. Among these, experiments with rifampicin have been the most successful (Hu, Zhang and Ochi 2002; Lai *et al.* 2002; Xu *et al.* 2002). Rifampicin (Fig. 1, **8**) is an inhibitor of RNAP, and accordingly rifampicin resistance is typically conferred by a mutation in the gene encoding its β-subunit, *rpoB* (Fig. 3B). When Hu *et al.* selected for rifampicin-resistant mutants (*rif*) in *S. lividans*, they observed that the mutant phenotype occurred concomitant with overproduction of Act (up to 10-fold), undecylprodigiosin (Red, up to 5-fold) and calcium-dependent antibiotic (CDA, fold-change not reported), three secondary metabolites that are minimally produced in the wt strain. Again, through mutant mapping and gene replacement studies, they showed that two classes of mutants could arise from rifampicin treatment: (1) those that provided resistance to rifampicin but did not enhance secondary metabolite production, and (2) those that provided resistance *and* upregulation of Act and Red. Interestingly, increased secondary metabolite production in the mutants was dependent on the location and nature of the amino acid substitution.

Perhaps the best application of ribosome engineering has been in the discovery of new cryptic metabolites (Hosaka *et al.* 2009). A screen of 1068 soil actinomycete isolates showed that 43% of non-antibiotic producing Streptomycetes and 6% of non-antibiotic-producing non-Streptomycetes acquired the ability to synthesize antibacterial compounds after a selection step that generated spontaneous *rif* or *str* mutants. Investigation of one of these isolates, which was determined to be closest to *S. mauvecolor* by 16S rRNA sequencing, led to the identification of a new cryptic antibiotic piperidamycin. Piperidamycin showed potent growth-inhibitory activity against Gram-positive bacteria, e.g. *Staphylococcus aureus*, *S. pyogenes* and *Enterococcus faecalis*, with MICs of 0.8–1.6 μg mL^{-1}. Thus, ribosome engineering can be used to improve production strains and induce silent biosynthetic gene clusters.

Mechanism

The onset of secondary metabolite production in Streptomycetes typically occurs at the end of exponential growth phase and the start of stationary phase, a period sometimes referred to as idiophase. A key regulatory system in this growth period is the stringent response, which is initiated by nutrient limitation (Starosta *et al.* 2014; Gaca, Colomer-Winter and Lemos 2015). When uncharged tRNAs, which accumulate under conditions of amino acid scarcity, bind to the A-site of the ribosome, RelA uses ATP and GTP to synthesize the hyperphosphorylated nucleotide ppGpp (guanosine-5′-diphosphate-3′-diphosphate). This so-called alarmone acts directly on RNAP, thus changing its transcriptional activity. A structure of the RNAP-ppGpp complex has been solved and a model for how ppGpp exerts its effect has been proposed at the molecular level (Artsimovitch *et al.* 2004). At the cellular level, production of ppGpp is associated with upregulation of stress response genes, such as those involved in amino acid uptake and biosynthesis as well as the induction of some secondary metabolites.

Upon observing the enhanced production of certain secondary metabolites using the *str* and *rif* phenotypes, Ochi and colleagues suspected that ppGpp was involved. However, numerous lines of evidence showed that the stringent response did not play a role (Ochi *et al.* 2004; Ochi 2007). Most importantly, direct measurement of the levels of ppGpp showed that they did not change significantly in the wt and mutant strains. In fact, the *str* strain showed slightly reduced levels of ppGpp. Further, *relA rif* and *relA str* double mutants, which cannot generate ppGpp, also exhibited stimulated production of secondary metabolites much like the single *str* and *rif* mutants. Thus, the *str* and *rif* mutants can circumvent ppGpp and stimulate production of certain secondary metabolites; that is, a 'stringent phenotype' can be induced in the absence of ppGpp.

The authors have proposed that the rifampicin-resistant *rpoB* mutant behaves like a 'stringent' RNAP, even in the absence of the alarmone ppGpp (Lai *et al.* 2002). Consistent with this idea, the *rif* mutants display a lower rate of RNA synthesis, a stringent phenotype, even in nutritionally rich medium. Further, the ppGpp-binding domain on the β-subunit of RNAP is close to the site of point mutations that render the *rif* phenotype. These mutations are only several angstroms removed from the active site, suggesting that they could have a major impact on the activity of RNAP. In the model proposed, the mutated RNAP provides rifampicin resistance and behaves like a stringent RNAP. It demonstrates an enhanced affinity for promoters, increasing expression of *actII-ORF4*, which encodes an *act*-pathway-specific positive transcriptional regulator, whose expression levels are directly correlated with Act production (Ochi and Hosaka 2013).

In the case of *str* mutants, ppGpp was also shown not to be required (Hosoya *et al.* 1998). By comparing a number of properties of wt and streptomycin-resistant ribosomes, the authors proposed that the mutant ribosome was structurally more stable under conditions of stress, such as amino acid starvation, and that it exhibited higher levels of protein synthesis in the stationary phase (Hosaka, Xu and Ochi 2006; Hosaka *et al.* 2009; Ochi and Hosaka 2013). In the case of Act biosynthesis, this increased protein synthesis activity in the stationary phase resulted in stimulated production of the positive transcriptional regulator *actII-ORF4*, as determined by western blot analysis, which led to augmented levels of the small molecule product.

Additional antibiotics have been used successfully for augmented secondary metabolite production by Ochi and coworkers. These include erythromycin and gentamicin, both of which target the ribosome (Chai *et al.* 2012; Imai *et al.* 2012). While further studies with these antibiotics are necessary, initial mechanistic experiments indicate that their mode of induction is different from those of streptomycin and rifampicin. It

seems likely that antibiotics with modes of action alternative to streptomycin and rifampicin will engage in new modes of induction of secondary metabolism. The ribosome, consisting of three rRNA molecules and over 50 proteins, provides an abundance of molecular targets that can be inhibited by specific antibiotics (Davies, Spiegelman and Yim 2006). The mechanistic details of how inhibition or mutation is linked to upregulated secondary metabolite production with other antibiotics will be of great interest.

A final interesting observation made by Ochi and coworkers was the induction of secondary metabolism in the presence of exogenous antibiotics but without development of a resistant phenotype. This was the case with tetracycline and lincomycin (Shima *et al.* 1996; Imai *et al.* 2015). It will be interesting to see what the mechanism of induced secondary metabolite production is in these non-resistant strains, a case that is akin to examples of goadsporin or promomycin in coculture experiments.

CHROMATIN REMODELING

Motivation

Chromatin remodeling is one of the newest techniques for induction of secondary metabolism. Its roots lie in a study from Keller and colleagues. While investigating the regulatory pathway of sterigmatocystin, a toxin produced by *Aspergillus nidulans*, Keller identified several mutant strains that showed suppressed production of the toxin (Butchko, Adams and Keller 1999). One of the mutations was in LaeA, a methyltransferase, implicating it as a regulator of sterigmatocystin production. In addition, LaeA had pleiotropic effects on secondary metabolism: its deletion blocked expression of several biosynthetic gene clusters, while its overexpression triggered penicillin and lovastatin production (Bok and Keller 2004). LaeA has homology to histone methyltransferases, which led Keller to propose a chromatin-based model of regulation. Indeed, deletion of *hdaA*, an *Aspergillus* histone deacetylase (HDAC), also led to increased production of two telomere-proximal secondary metabolite gene clusters. In contrast, transcription of a telomere-distal cluster was unchanged (Shwab *et al.* 2007). These studies clearly implicated a chromatin modification-based regulatory system in *Aspergillus* (Palmer and Keller 2010). Building on these results, Keller and Cichewicz have provided further examples of chromatin remodeling by mimicking these epigenetically altered genotypes via supplementation of fungal cultures with HDAC and DNA methyltransferase inhibitors.

Applications

In the study demonstrating the importance of HdaA in regulating secondary metabolism, Keller and colleagues included a small set of proof-of-concept experiments that laid the groundwork for chromatin remodeling. Trichostatin A, a class 1/2 HDAC inhibitor, was added to *Alternaria alternata* and *Penicillium expansum*. This treatment caused an increase in the production of multiple unidentified small molecules (Shwab *et al.* 2007). Cichewicz expanded on these studies, subjecting a set of 12 fungi to a library of DNA methyltransferase (DNMT) and HDAC inhibitors at various concentrations. A total of 11 of the 12 strains could be induced by at least one member of the library to increase their levels or diversity of secondary metabolism. Two of the fungi were studied further. *Cladosporium cladosporioides* was stimulated by 5-azacytidine (5-AC, Fig. 1, **9**), a DNMT inhibitor, to produce several cryptic oxylipins, or by suberoylanilide hydroxamic acid (SAHA, Fig. 1, **10**), an HDAC inhibitor, to produce a series of cryptic perylenequinones, including both known and novel cladochrome analogs (Fig. 4). The second fungus, a *Diatrype* species, was induced by 5-AC to produce two new cryptic polyketides, lunalides A and B (Fig. 4B) (Williams *et al.* 2008).

This approach was also successful in *A. niger*, which could be induced by SAHA to produce the new cryptic metabolite nygerone A (Fig. 4A) (Henrikson *et al.* 2008). Similarly, 5-AC or SAHA treatment of *Alternaria* sp. led to the production of several cryptic mycotoxins including alternariol, altenusin and tenuazonic acid, and increased production of altertoxin II (Sun *et al.* 2012). A study of the global effects of 5-AC and SAHA on the transcription of 55 polyketide synthase (PKS), non-ribosomal peptide synthetase (NRPS) or PKS/NRPS gene clusters was consistent with the results above (Fisch *et al.* 2009). It showed that ~70% of these biosynthetic gene clusters were inactive when the fungus was grown using standard laboratory culture conditions, and that addition of 5-AC or SAHA induced transcription of all but seven of these (Fisch *et al.* 2009). Oberlies and coworkers took a slightly different approach to this method, using the proteasome inhibitor bortezomib to induce a filamentous fungus to produce cryptic metabolite **11** (Fig. 1) (VanderMolen *et al.* 2014). While mode-of-action restrictions largely limit this method's application to fungi, the McArthur group was able to apply it in *Streptomyces coelicolor* (Moore *et al.* 2012). Using qPCR, the group showed that the HDAC inhibitor sodium butyrate induced five cryptic pathways in Streptomycetes. They also performed activity-based assays on *Streptomyces* sp. KY5 and *Pseudonocardia* sp. P1 and saw induced activity against *Candida albicans* when the bacteria were cultured with sodium butyrate (Moore *et al.* 2012).

Mechanism

Fungal DNA, like that of higher eukaryotes, is organized onto histones. Histones compact the DNA into chromatin and regulate replication and transcription. There are two states of chromatin: a 'closed' state, called heterochromatin, is more densely packed and therefore transcriptionally silent. In contrast, the 'open' state, called euchromatin, is more loosely packed and transcriptionally active (Brosch, Loidl and Graessle 2008; Gacek and Strauss 2012).

Fungi can transition portions of chromatin between these two states through various post-translational modifications, the most well studied of which is histone acetylation. Unmodified lysine is predominately cationic, allowing it to bind to DNA; acetylation abrogates the positive charge on lysine, occluding DNA binding. Accordingly, more highly acetylated histones are generally more loosely packed and transcriptionally accessible (Fig. 4A) (Cichewicz 2010). Further, the DNA base cytosine can be methylated to form 5-methylcytosine. This modification can have different effects on transcription depending on specific contexts, but often results in gene silencing (Fig. 4B) (Suzuki and Bird 2008). Fungi are primed for chromatin remodeling because of the organization of their genome: most fungal secondary metabolite genes are grouped together in locations that tend to be near the telomeres of their chromosomes (Keller, Turner and Bennet 2005; Yu and Keller 2005). As a result, histone acetylation and DNA methylation have a large impact on the transcription of these loci (Bok and Keller 2004; Shwab *et al.* 2007; Palmer and Keller 2010; Gacek and Strauss 2012). Targeting the enzymes that regulate these markers is a straightforward way to probe the effects of different epigenetic conditions. Thus, DNMT and HDAC inhibitors serve as effective elicitors of fungal secondary

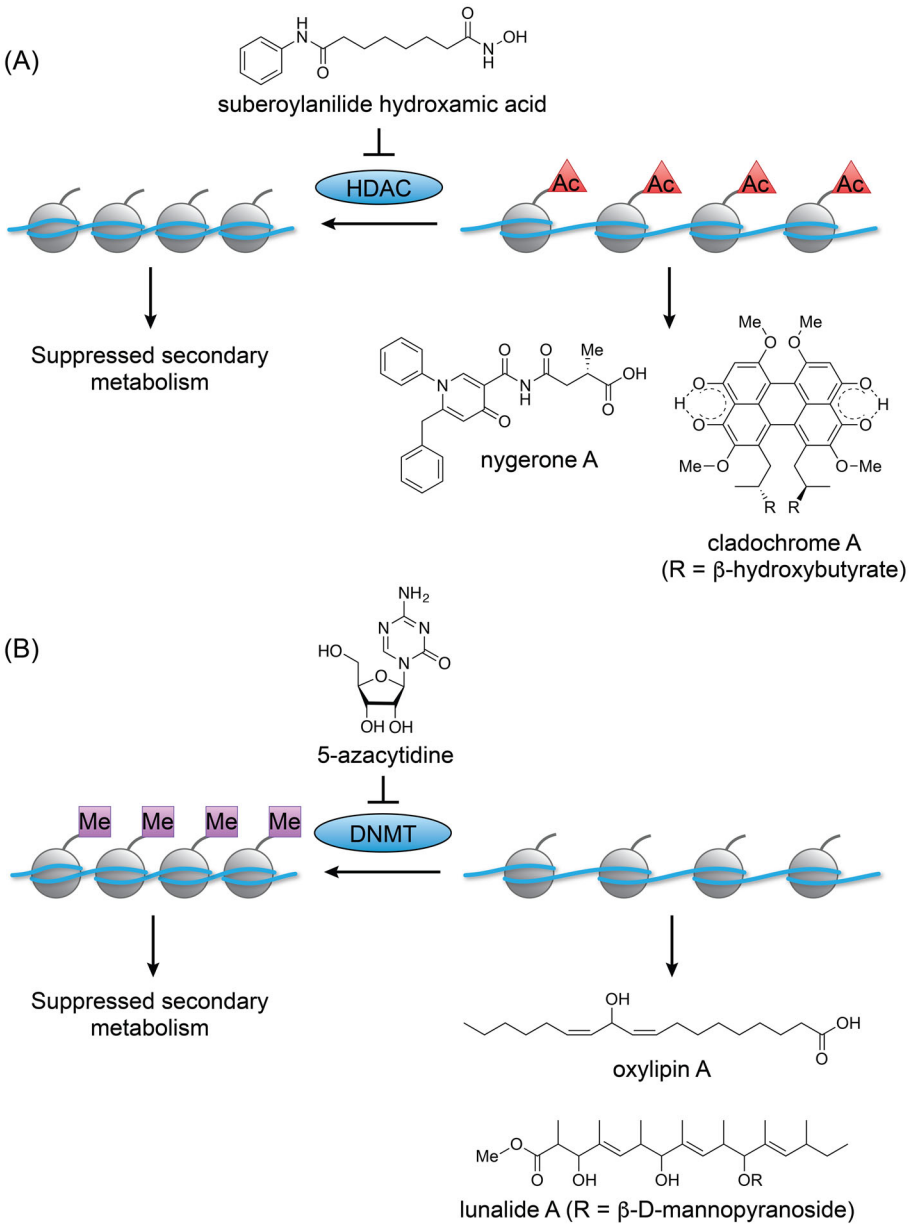

Figure 4. Mechanisms underlying chromatin remodeling. (**A**) Treatment with the HDAC inhibitor SAHA results in increased histone acetylation and consequently increased transcription of biosynthetic gene clusters. (**B**) Treatment with the DNMT inhibitor 5-AC results in decreased DNA methylation, altering the transcription of various biosynthetic gene clusters.

metabolite. The use of proteasome inhibitors takes advantage of the same underlying mechanism in an indirect manner: proteasomes degrade many proteins, including several transcriptional regulators (VanderMolen *et al.* 2014).

Because bacterial DNA is not organized onto histones, it is somewhat surprising that McArthur and coworkers had success using this method in Streptomycetes (Moore *et al.* 2012). The authors explained their results by citing a parallel to chromatin organization: the bacterial genome is compacted by nucleoid-associated proteins, RNAs and differential supercoiling, which could lead to differential compaction for certain genes. In addition, bacteria have their own versions of HDAC proteins (Lombardi *et al.* 2011). The mechanism for how these bacterial HDAC-like enzymes function is still unclear, so the pathway for the epigenetic approach in bacteria ultimately awaits further clarification (Moore *et al.* 2012). They might indeed act on nucleoid proteins, though other proposed targets include polyamines, acetoin and regulators of primary metabolism (Leipe and Landsman 1997).

Chromatin remodeling has proven to be a straightforward, low-cost, effective means of activating cryptic gene clusters in fungi (Gacek and Strauss 2012). Inherent limitations result from its mechanism of action, which largely (theoretically) confine it to fungi and to telomere proximal biosynthetic gene clusters. Its recent application in Streptomycetes indicates that it may be a more general method than previously thought, but is currently the only example of chromatin remodeling outside of fungi. We eagerly await further assessments of this method's scope, as well as investigation of its mechanism in bacteria.

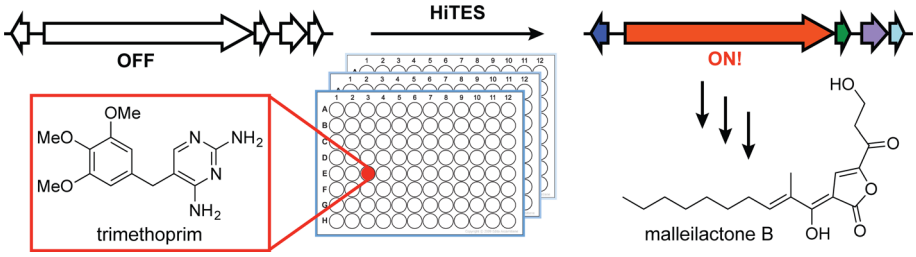

Figure 5. Workflow for HiTES. A library of small molecules is screened to identify elicitors that induce expression of a cryptic gene cluster. The read-out can be pigment production or a genetic reporter via transcriptional or translational reporter fusion constructs. In *B. thailandensis*, trimethoprim was identified as an elicitor of malleilactone production using this approach.

HiTES

Motivation

The preceding sections show that antibiotics are often elicitors of secondary metabolism in fungi and bacteria. However, which antibiotic, or more generally small molecule, serves as an inducer for a specific strain or biosynthetic gene cluster, needs to be determined experimentally. Consider the example of goadsporin (Onaka *et al.* 2001). It was shown to be a *Streptomyces*-specific antibiotic at high concentrations but to elicit secondary metabolism at subinhibitory levels. However, its mode of induction was likely independent of its mode of growth inhibition, as a number of other toxins tested—including streptomycin, kanamycin, thiostrepton, bacitracin and gramicidin D—did not stimulate secondary metabolism. In this regard, bioinformatic tools are of little help: while we are increasingly adept at providing an *in silico* analysis of a biosynthetic gene cluster and predicting the class of molecule that might result, it is extremely difficult, if not impossible, to predict which small molecule signal, if any, will activate it. To address this shortcoming and identify small molecule elicitors for a chosen gene cluster, high-throughput methods have been developed, an approach that we refer to as HiTES (**hi**gh-**t**hroughput **e**licitor **s**creens).

Applications

There are three key components involved in this approach: the choice of microorganism, read-out and small molecule library to be screened. The first rendition of HiTES was carried out by Nodwell and coworkers (Craney *et al.* 2012). They used *Streptomyces coelicolor* based on the historical importance and fruitfulness of this strain and because it synthesizes the blue polyketide actinorhodin and the red prodiginines, the production of which can be rapidly monitored. The Canadian Compound Collection, which contains 30 569 small molecules, was screened for compounds that increased actinorhodin production in *S. coelicolor*. From this screen, 112 compounds were identified as hits, and a subset of these containing four related compounds (named the 'ARC2 series') were used for further studies. In addition to upregulating actinorhodin synthesis 2- to 5-fold, ARC2 (Fig. 1, **12**) was able to stimulate 3-fold overproduction of the germicidins, while decreasing yields of the daptomycin-like CDA and prodiginines approximately 2-fold. Thus, ARC2 elicitors displayed pleiotropic effects on secondary metabolism. ARC2 is structurally similar to triclosan (Fig. 1, **13**), which provided some clues regarding the mode of induction by this elicitor (see below). Strikingly, ARC2 and triclosan, which also induces actinorhodin synthesis, both displayed hormetic properties, and modulated secondary metabolism only at subinhibitory concentrations.

Spurred by the results with ARC2, the authors also investigated its effects on other actinomycetes and found it to serve as a potent general elicitor: it altered the secondary metabolome of *Kutzneria* sp., *S. pristinaespiralis* and *S. peucetius*, where it induced production of cryptic metabolites. A derivative of ARC2, called Cl-ARC (Fig. 1, **14**), has recently been applied to 50 additional *Streptomyces*, resulting in at least one induced compound in a majority of the strains for a total of 216 cryptic metabolites (Pimentel-Elardo *et al.* 2015).

The other example of HiTES took a more general approach that can be applied to non-pigmented secondary metabolites. We demonstrated the effectiveness of HiTES in *Burkholderia thailandensis*, which was chosen for its importance as a model strain for the *Pseudomallei* group pathogens, genetic tractability and wealth of silent biosynthetic gene clusters (Liu and Cheng 2014; Seyedsayamdost 2014). In place of a phenotypic screen, a *lacZ* translational fusion to an essential gene within a selected biosynthetic gene cluster was employed as a reporter system. We chose the *mal* gene cluster, which synthesizes the cryptic virulence factor malleilactone, a potent inhibitor of *Caenorhabditis elegans* growth (Fig. 5). A 640-member library of bioactive small molecules was screened by monitoring the *lacZ* activity induced by each member of the compound library. From 640 compounds, nine elicitors were identified, which upregulated expression of the *mal* cluster (Fig. 5). Intriguingly, all nine elicitors were clinical antibiotics: trimethoprim, piperacilllin, ceftazidime, cefotaxime and five members of the fluoroquinolone family. At high concentrations, all of these antibiotics killed *B. thailandensis*, whereas subinhibitory levels served as potent inducers of the *mal* cluster. The top two elicitors, trimethoprim and piperacillin, showed a 5- to 45-fold upregulation of selected genes in the *mal* gene cluster, as determined by RT-qPCR. At optimal concentration, a ~150-fold induction of malleilactone was observed with trimethoprim.

Further investigations showed that trimethoprim served as a global activator of secondary metabolism in *B. thailandensis* (Okada *et al.* 2016). Production of at least five secondary metabolite families was induced as demonstrated by qPCR and/or metabolic profiling studies. These include thailandamide (8- to 36-fold overproduction depending on the analog), burkholdac (3-to 4-fold), 4-hydroxy-3-methyl-2-alkylquinolines (~7-fold) and capistruin lasso peptides (not produced under normal growth), as well as a family of new cryptic metabolites called acybolins that has recently been structurally elucidated. Additional effects of trimethoprim on the secondary metabolic output of *B. thailandensis* have also been characterized.

Mechanism

A great strength and weakness of HiTES is that it is mechanism agnostic. It does not rely on a specific hypothesis for the mode of action of the elicitors identified. As a consequence, it is impossible to generalize its mode of induction. While this makes

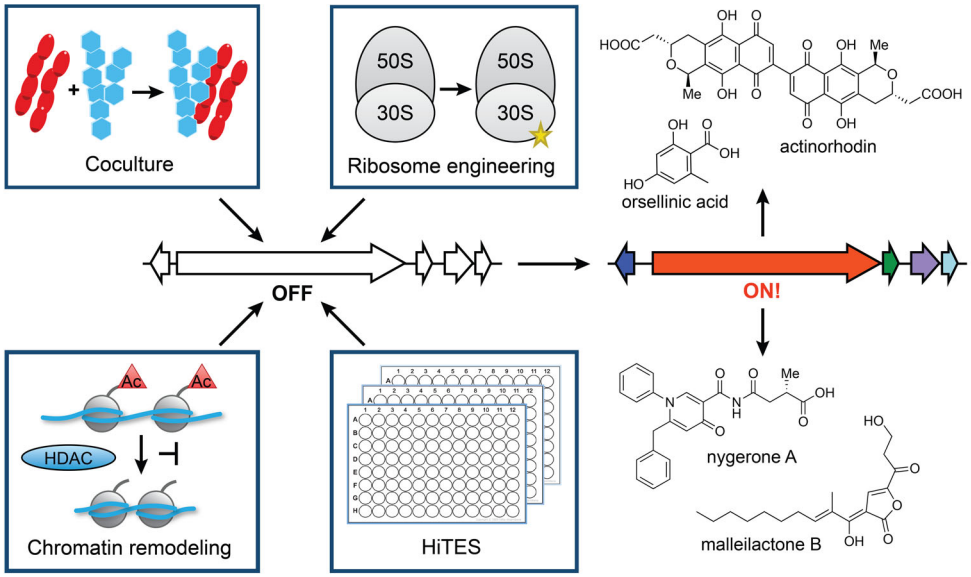

Figure 6. Summary of approaches discussed in this review for activation of silent biosynthetic gene clusters using small molecules.

assigning a specific mechanism to a given elicitor more difficult, it also allows for the discovery of elicitors that operate through unforeseen or unprecedented modes of induction, thus broadening the scope of gene clusters that can be activated.

In the case of the ARC2 compound series, a molecular target was proposed based on the series' structural similarity to triclosan, a known inhibitor of fatty acid synthesis that targets the FabI enoyl reductase (Craney *et al.* 2012). When tested against *S. coelicolor,* triclosan recapitulated the pigmentation induction of the ARC2 compounds. In addition, an *S. coelicolor* strain expressing a triclosan-resistant FabI paralog from *Pseudomonas aeruginosa* showed significantly reduced pigmentation compared to the control strain. Finally, biochemical assays of FabI with and without the ARC2 compounds confirmed that it could be inhibited by the series. Based on these results, the authors hypothesized that inhibition of FabI might redirect precursors, such as acetyl-CoA and malonyl-CoA, that are shared between fatty acid and polyketide synthesis, increasing the yield of the polyketide natural products. This link between fatty acid and polyketide biosynthesis, the authors proposed, helps set the upper limit on polyketide yields. Recent results show that the regulatory mechanism by ARC2 might be more complex and future studies will likely shed more light on this issue (Ahmed *et al.* 2013).

A mechanism for trimethoprim and other inducers found for *B. thailandensis* still remains to be determined. Based on the known antibiotic activity of the identified elicitors, it seems likely that their effects are mediated through stress response mechanisms. Trimethoprim has been shown to induce stress response in *Escherichia coli* (Khan and Yamazaki 1972). In addition, beta-lactam resistance in *Staphylococcus aureus* is mediated via stress response-related pathways (Aedo and Tomasz 2016). As discussed earlier in the context of ribosome engineering, cellular stress can alter the activity of the ribosome and thus induce secondary metabolism at the start of stationary phase. It is likely that the subinhibitory concentrations of antibiotics used in our study initiated stress response in *B. thailandensis,* resulting in the pleiotropic stimulation of secondary metabolism. Nonetheless, an alternative mechanism cannot be excluded at this point. For example, results by Davies (Goh *et al.* 2002) have shown that erythromycin-mediated transcriptional modulation occurred to the same extent in both wt and mutants defective in a variety of stress response pathways. Hu *et al.* reached a similar conclusion when examining the transcriptional changes induced by the antibiotic cecropin in *E. coli* O157 (Hong *et al.* 2003).

HiTES is a versatile, theoretically general method for activation of cryptic gene clusters. While relatively new, it promises to uncover new natural products and identify elicitors that operate through unprecedented mechanisms of activation, thus providing insights into the still poorly-understood regulatory pathways of secondary metabolite production.

CONCLUSIONS

Recent technological advances have rejuvenated the field of natural products discovery, unearthing a vast untapped cache of silent biosynthetic gene clusters. Current efforts to take advantage of this new knowledge are limited by a lack of understanding of how secondary metabolism is regulated and therefore how the clusters might be activated. In this review, we have summarized the methods that have been developed to induce secondary metabolism using chemical approaches as well as the underlying biological mechanisms (Fig. 6). While the methods described in the preceding sections have distinct rationales, two underlying commonalities can be identified. The first key theme is the existence of hormesis—an inherent concentration dependence in the induced response to growth-inhibitory molecules (Davies 2006). Nearly 65 years ago, Hessayon provided perhaps the first report of hormesis by showing that the antibiotic trichothecin, produced in very small quantities by *Trichothecium roseum,* produced stimulatory effects on the growth of the plant pathogen *Fusarium oxysporum*, whereas higher concentrations had toxic effects (Hessayon 1953). This phenomenon, recently emphasized and shown to be prevalent by Davies and colleagues, was observed in numerous examples above. We have highlighted these instances of hormesis in the preceding sections, where subtoxic concentrations of antibiotics elicited sporulation, development or secondary metabolite biosynthesis, while higher titers caused cell death.

The second common theme is that growth inhibition is in many cases key to stimulation of secondary metabolism. Numerous examples demonstrate that microorganisms often respond to growth-inhibitory molecules by producing their own growth-inhibitory molecules. This observation has important repercussions. First, it suggests that old antibiotics may be used to discover new ones. In strong support of this proposition are a number of results described above, including (a) multiple examples of antibiotic synthesis in response to growth-inhibitory molecules in coculture assays; (b) the discovery of piperidamycin using streptomycin and rifampicin in ribosome engineering; (c) chromatin remodeling studies, where SAHA and other chromatin modulators display growth-inhibitory properties and elicit bioactive metabolite production; and (d) early results with HiTES, where antibiotics have been identified as inducers of cryptic virulence factors. Second, the observation above provides hints regarding how bacteria perceive antibiotics; that is, they give us clues about antibiotics' natural roles. That bacteria in many cases respond to antibiotics with antibiotics could point to the existence of chemical warfare among neighboring colonies. However, Waksman, Davies and others have pointed out that naturally occurring concentrations of antibiotics are not high enough to serve as biocides in the environment (Waksman 1961; Davies 2006; Yim, Wang and Davies 2007). As such, the exchange of antibiotics should in most cases be interpreted as an interaction or communication. That is, in the wild, antibiotics usually serve as mediators of microbial interactions and shape multispecies communities in their microenvironment. What would be the role of antibiotic resistance in this context? In an anthropomorphic sense, resistance may be akin to putting on headphones and not participating in a conversation.

Additional insights regarding the roles of some antibiotics may be gained by considering the following: studies by Ochi and a number of coculture experiments show that the response of Streptomycetes to nutrient limitation is often similar to their response to antibiotic treatment. This may bring another role for antibiotics to the fore, namely that of deterrents or warning signals. A nutrient-poor environment is an undesirable one, and the similar responses of some bacteria to antibiotics would lead one to conclude that antibiotics can act as deterrents. There are parallels from a molecular point of view as well: nutrient limitation triggers the stringent response, which leads to an altered RNAP specificity via the molecule ppGpp. Similarly, antibiotic treatment or resistance can lead to activation of the stringent response or even an RNAP that acts as if 'stringent' even in the absence of ppGpp. The idea of inducible defense is well accepted in the plant research community. Phytoalexins are inducible defense metabolites produced by plants in response to certain threats. Might some bacterially produced antibiotics serve as an inducible defense mechanism—say, as bacterioalexins? Note that this does not imply that they are necessarily bactericidal, but simply that they may be inducible responses to undesired conditions.

Ultimately, it is difficult to ascribe a specific role to antibiotics simply because there is no single answer. The role of an antibiotic depends on the specific antibiotic and the nature of the recipient cell. To the recently discovered strain *Amycolatopsis* sp. AA4, which is resistant to 15 antibiotics, including glycopeptides, vancomycin plays no role at all (D'Costa *et al*. 2006). To organisms that do not have the requisite resistance genes, vancomycin may serve as a threat or a signal and therefore elicit complex changes in gene expression. However, some antibiotics, which kill at concentrations as low as a few molecules per cell, such as enediynes or bleomycins, should be considered chemical warfare agents (Povirk 1996). Thus, depending on the antibiotic and the recipient, the roles may vary from no function at all, to signals that elicit changes in gene expression patterns, to inducers of stress and to cell death-causing toxins. It is especially important to consider the role of the recipient cell. Because there is a concentration gradient of an antibiotic emanating from the producing strain, the neighboring cell will always initially sense subinhibitory concentrations of most antibiotics. Transcriptional regulation in bacteria is rapid and thus the recipient could activate antibiotic production or sporulation pathways to redirect or entirely evade the conversation.

The foregoing discussion shows that this research field, referred to as small molecule biology by Davies, is a fascinating one. Small molecules can certainly be used to probe and activate the store of silent biosynthetic gene clusters that have been found in microbial genomes. It is clear that bacteria have evolved complex response mechanisms to antibiotics. Much like quorum-sensing molecules, subinhibitory concentrations of antibiotics affect the expression of a set of particular genes, an antibiotic-specific regulon. Further investigations of this regulon will deepen our understanding of small molecule biology and, by stimulating secondary metabolism, provide additional bioactive molecules for use in human health and beyond.

ACKNOWLEDGEMENTS

The authors thank Clarissa Forneris and Paul Rosen for comments on the manuscript, and Marcus Gibson for assistance with figures.

FUNDING

The authors thank the National Institutes of Health (DP2-124786-01 to MRS) and the Searle Scholars Program of the Kinship Foundation (to MRS) for generous support of our work.

Conflict of interest. None declared.

REFERENCES

Aedo S, Tomasz A. Role of the stringent stress response in the antibiotic resistance phenotype of methicillin-resistant Staphylococcus aureus. *Antimicrob Agents Ch* 2016;**60**:2311–7.

Ahmed S, Craney A, Pimentel-Elardo SM *et al*. A synthetic, species-specific activator of secondary metabolism and sporulation in *Streptomyces coelicolor*. *Chembiochem* 2013;**14**:83–91.

Amano S, Morota T, Kano Y *et al*. Promomycin, a polyether promoting antibiotic production in *Streptomyces* spp. *J Antibiot* 2010;**63**:486–91.

Amano S, Sakurai T, Endo K *et al*. A cryptic antibiotic triggered by monensin. *J Antibiot* 2011;**64**:703.

Artsimovitch I, Patlan V, Sekine S *et al*. Structural basis for transcription regulation by alarmone ppGpp. *Cell* 2004;**117**:299–310.

Bentley SD, Chater KF, Cerdeño-Tárraga A-M *et al*. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* 2002;**417**:141–7.

Berdy J. Bioactive microbial metabolites. *J Antibiot* 2005;**58**:1–26.

Bode HB, Bethe B, Höfs S *et al*. Big effects from small changes: possible ways to explore nature's chemical diversity. *Chembiochem* 2002;**3**:619–27.

Bok JW, Keller NP. LaeA, a Regulator of secondary metabolism in *Aspergillus* spp. *Eukaryot Cell* 2004;**3**:527–35.

Brosch G, Loidl P, Graessle S. Histone modifications and chromatin dynamics: a focus on filamentous fungi. *FEMS Microbiol Rev* 2008;**32**:409–39.

Butchko RA, Adams TH, Keller NP. *Aspergillus nidulans* mutants defective in stc gene cluster regulation. *Genetics* 1999;**153**:715–20.

Buzzini P. Batch and fed-batch carotenoid production by *Rhodotorula glutinis-Debaryomyces castellii* co-cultures in corn syrup. *J Appl Microbiol* 2001;**90**:843–7.

Chai YJ, Cui CB, Li CW *et al.* Activation of the dormant secondary metabolite production by introducing gentamicin-resistance in a marine-derived *Penicillium purpurogenum* G59. *Mar Drugs* 2012;**10**:559–82.

Chiang YM, Chang SL, Oakley BR *et al.* Recent advances in awakening silent biosynthetic gene clusters and linking orphan clusters to natural products in microorganisms. *Curr Opin Chem Biol* 2011;**15**:137–43.

Cichewicz RH. Epigenome manipulation as a pathway to new natural product scaffolds and their congeners. *Nat Prod Rep* 2010;**27**:11–22.

Clatworthy AE, Pierson E, Hung DT. Targeting virulence: a new paradigm for antimicrobial therapy. *Nat Chem Biol* 2007;**3**: 541–8.

Cragg GM, Newman DJ. Natural products: a continuing source of novel drug leads. *Biochim Biophys Acta* 2013;**1830**: 3670–95.

Craney A, Ozimok C, Pimentel-Elardo SM *et al.* Chemical perturbation of secondary metabolism demonstrates important links to primary metabolism. *Chem Biol* 2012;**19**:1020–7.

Cueto M, Jensen PR, Kauffman C *et al.* Pestalone, a new antibiotic produced by a marine fungus in response to bacterial challenge. *J Nat Prod* 2001;**64**:1444–6.

Curtis TP, Sloan WT, Scannell JW. Estimating prokaryotic diversity and its limits. *P Natl Acad Sci USA* 2002;**99**:10494–9.

D'Costa VM, McGrann KM, Hughes DW *et al.* Sampling the antibiotic resistome. *Science* 2006;**311**:374–7.

Davies J. Are antibiotics naturally antibiotics? *J Ind Microbiol Biot* 2006;**33**:496–9.

Davies J, Spiegelman GB, Yim G. The world of subinhibitory antibiotic concentrations. *Curr Opin Microbiol* 2006;**9**:445–53.

Fourati-Ben Fguira L, Smaoui S, Karray-Rebai I *et al.* The antifungal activity of the terrestrial *Streptomyces* US80 strain is induced by heat-killed fungi. *Biotechnol J* 2008;**3**:1058–66.

Fisch KM, Gillaspy AF, Gipson M *et al.* Chemical induction of silent biosynthetic pathway transcription in *Aspergillus niger*. *J Ind Microbiol Biot* 2009;**36**:1199–213.

Fischbach MA, Walsh CT. Antibiotics for emerging pathogens. *Science* 2009;**325**:1089–93.

Gaca AO, Colomer-Winter C, Lemos JA. Many means to a common end: the intricacies of (p)ppGpp metabolism and its control of bacterial homeostasis. *J Bacteriol* 2015;**197**:1146–56.

Gacek A, Strauss J. The chromatin code of fungal secondary metabolite gene clusters. *Appl Microbiol Biot* 2012;**95**:1389–404.

Gaudêncio SP, Pereira F. Dereplication: racing to speed up the natural products discovery process. *Nat Prod Rep* 2015;**32**:779–810.

Goh EB, Yim G, Tsui W *et al.* Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics. *P Natl Acad Sci USA* 2002;**99**:17025–30.

Henrikson JC, Hoover AR, Joyner PM *et al.* A chemical epigenetics approach for engineering the *in situ* biosynthesis of a cryptic natural product from *Aspergillus niger*. *Org Biomol Chem* 2008;**7**:435–8.

Hessayon DG. Fungitoxins in the soil: I. Historical. *Soil Sci* 1953;**75**:317–28.

Hong RW, Shchepetov M, Weiser JN *et al.* Transcriptional profile of the *Escherichia coli* response to the antimicrobial peptide cecropin A. *Antimicrob Agents Chemother* 2003;**47**:1–6.

Hosaka T, Ohnishi-Kameyama M, Muramatsu H *et al.* Antibacterial discovery in actinomycetes strains with mutations in RNA polymerase or ribosomal protein S12. *Nat Biotechnol* 2009;**27**:462–4.

Hosaka T, Xu J, Ochi K. Increased expression of ribosome recycling factor is responsible for the enhanced protein synthesis during the late growth phase in an antibiotic-overproducing *Streptomyces coelicolor* ribosomal rpsL mutant. *Mol Microbiol* 2006;**61**:883–97.

Hosoya Y, Okamoto S, Muramatsu H *et al.* Acquisition of certain streptomycin-resistant (*str*) mutations enhances antibiotic production in bacteria. *Antimicrob Agents Ch* 1998;**42**: 2041–7.

Hu H, Zhang Q, Ochi K. Activation of antibiotic biosynthesis by specified mutations in the *rpoB* gene (encoding the RNA polymerase beta subunit) of *Streptomyces lividans*. *J Bacteriol* 2002;**184**:3984–91.

Iakovleva EP, Sokolova EN. Dissociation of a *Candida tropicalis* culture and its capacity to stimulate levorin synthesis when cultured together with *Actinomyces levoris*. *Antibiotiki* 1978;**23**:199–203.

Ikeda H, Ishikawa J, Hanamoto A *et al.* Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*. *Nat Biotechnol* 2003;**21**:526–31.

Imai Y, Fujiwara T, Ochi K *et al.* Development of the ability to produce secondary metabolites in *Streptomyces* through the acquisition of erythromycin resistance. *J Antibiot* 2012;**65**:323–6.

Imai Y, Sato S, Tanaka Y *et al.* Lincomycin at subinhibitory concentrations potentiates secondary metabolite production by *Streptomyces* spp. *Appl Environ Microb* 2015;**81**:3869–79.

Keller NP, Turner G, Bennet JW. Fungal secondary metabolism - from biochemistry to genomics. *Nat Rev Microbiol* 2005;**3B**:937–47.

Khan SR, Yamazaki H. Trimethoprim-induced accumulation of guanosine tetraphosphate (ppGpp) in *Escherichia coli*. *Biochem Bioph Res Co* 1972;**48**:169–74.

Kolter R, van Wezel GP. Goodbye to brute force in antibiotic discovery? *Nat Microbiol* 2016;**1**:15020.

Lai C, Xu J, Tozawa Y *et al.* Genetic and physiological characterization of *rpoB* mutations that activate antibiotic production in *Streptomyces lividans*. *Microbiology* 2002;**148**:3365–73.

Leipe DD, Landsman D. Histone deacetylases, acetoin utilization proteins and acetylpolyamine amidohydrolases are members of an ancient protein superfamily. *Nucleic Acids Res* 1997;**25**:3693–7.

Lombardi PM, Angell HD, Whittington DA *et al.* Structure of prokaryotic polyamine deacetylase reveals evolutionary functional relationships with eukaryotic histone deacetylases. *Biochemistry* 2011;**50**:1808–17.

Long RA, Azam F. Antagonistic interactions among marine pelagic bacteria. *Appl Environ Microb* 2001;**67**:4975–83.

Liu X, Cheng YQ. Genome-guided discovery of diverse natural products from *Burkholderia* sp. *J Ind Microbiol Biot* 2014;**41**:275–84.

Marmann A, Aly AH, Lin W *et al.* Co-cultivation—a powerful emerging tool for enhancing the chemical diversity of microorganisms. *Mar Drugs* 2014;**12**:1043–65.

Mearns-Spragg A, Bregu M, Boyd KG *et al.* Cross-species induction and enhancement of antimicrobial activity produced by epibiotic bacteria from marine algae and invertebrates, after exposure to terrestrial bacteria. *Lett Appl Microbiol* 1998;**27**:142–6.

Meng L, Li M, Yang SH *et al.* Intracellular ATP levels affect secondary metabolite production in *Streptomyces* spp. *Biosci Biotech Bioch* 2011;**75**:1576–81.

Miller SJ, Clardy J. Natural products: Beyond grind and find. *Nat Chem* 2009;**1**:261–3.

Mohamed A, Nguyen CH, Mamitsuka H. Current status and prospects of computational resources for natural product dereplication: a review. *Brief Bioinform* 2016;**17**:309–21.

Moore JM, Bradshaw E, Seipke RF *et al.* Use and discovery of chemical elicitors that stimulate biosynthetic gene clusters in streptomyces bacteria. *Methods Enzymol* 2012;**517**:367–85.

Nett M, Ikeda H, Moore BS. Genomic basis for natural product biosynthetic diversity in the actinomycetes. *Nat Prod Rep* 2009;**26**:1362–84.

Newman DJ, Cragg GM. Natural product scaffolds as leads to drugs. *Future Med Chem* 2009;**1**:1415–27.

Newman DJ, Cragg GM. Natural products as sources of new drugs from 1981 to 2014. *J Nat Prod* 2016;**79**:629–61.

Nützmann HW, Reyes-Dominguez Y, Scherlach K *et al.* Bacteria-induced natural product formation in the fungus *Aspergillus nidulans* requires Saga/Ada-mediated histone acetylation. *P Natl Acad Sci USA* 2011;**108**:14282–7.

Ochi K. From microbial differentiation to ribosome engineering. *Biosci Biotech Bioch* 2007;**71**:1373–86.

Ochi K, Hosaka T. New strategies for drug discovery: activation of silent or weakly expressed microbial gene clusters. *Appl Microbiol Biot* 2013;**97**:87–98.

Ochi K, Okamoto S, Tozawa Y *et al.* Ribosome engineering and secondary metabolite production. *Adv Appl Microbiol* 2004;**56**:155–84.

Oh D-C, Jensen PR, Kauffman CA *et al.* Libertellenones A–D: induction of cytotoxic diterpenoid biosynthesis by marine microbial competition. *Bioorg Med Chem* 2005;**13**:5267–73.

Okada BK, Wu Y, Mao D *et al.* Mapping the trimethoprim-induced secondary metabolome of *Burkholderia thailandensis*. *ACS Chem Biol* 2016;**11**:2124–30.

Ola ARB, Thomy D, Lai D *et al.* Inducing secondary metabolite production by the endophytic fungus *Fusarium tricinctum* through coculture with *Bacillus subtilis*. *J Nat Prod* 2013;**76**:2094–9.

Oliynyk M, Samborskyy M, Lester JB *et al.* Complete genome sequence of the erythromycin-producing bacterium *Saccharopolyspora erythraea* NRRL23338. *Nat Biotechnol* 2007;**25**:447–53.

Onaka H, Tabata H, Igarashi Y *et al.* Goadsporin, a chemical substance which promotes secondary metabolism and morphogenesis in streptomycetes. I. Purification and characterization. *J Antibiot* 2001;**54**:1036–44.

Palmer JM, Keller NP. Secondary metabolism in fungi: does chromosomal location matter? *Curr Op Microbiol* 2010;**13**:431–6.

Patterson GML, Bolis CM. Fungal cell-wall polysaccharides elicit an antifungal secondary metabolite (Phytoalexin) in the cyanobacterium *Scytonema ocellatum*. *J Phycol* 1997;**33**:54–60.

Pettit RK. Mixed fermentation for natural product drug discovery. *Appl Microbiol Biot* 2009;**83**:19–25.

Pimentel-Elardo SM, Sørensen D, Ho L *et al.* Activity-independent discovery of secondary metabolites using chemical elicitation and cheminformatic inference. *ACS Chem Biol* 2015;**10**:2616–23.

Povirk LF. DNA damage and mutagenesis by radiomimetic DNA-cleaving agents: bleomycin, neocarzinostatin and other enediynes. *Mutat Res* 1996;**355**:71–89.

Projan SJ. Why is big Pharma getting out of antibacterial drug discovery? *Curr Opin Microbiol* 2003;**6**:427–30.

Rigali S, Titgemeyer F, Barends S *et al.* Feast or famine: the global regulator DasR links nutrient stress to antibiotic production by *Streptomyces*. *EMBO Rep* 2008;**9**:670–5.

Rutledge PJ, Challis GL. Discovery of microbial natural products by activation of silent biosynthetic gene clusters. *Nat Rev Microbiol* 2015;**13**:509–23.

Santos CN, Stephanopoulos G. Combinatorial engineering of microbes for optimizing cellular phenotype. *Curr Opin Chem Biol* 2008;**12**:168–76.

Schroeckh V, Scherlach K, Nützmann H-W *et al.* Intimate bacterial–fungal interaction triggers biosynthesis of archetypal polyketides in *Aspergillus nidulans*. *P Natl Acad Sci USA* 2009;**106**:14558–63.

Seyedsayamdost MR. High-throughput platform for the discovery of elicitors of silent bacterial gene clusters. *P Natl Acad Sci USA* 2014;**111**:7266–71.

Seyedsayamdost MR, Case RJ, Kolter R *et al.* The Jekyll-and-Hyde chemistry of *Phaeobacter gallaeciensis*. *Nat Chem* 2011a;**3**:331–5.

Seyedsayamdost MR, Traxler MF, Zheng SL *et al.* Structure and biosynthesis of amychelin, an unusual mixed-ligand siderophore from *Amycolatopsis* sp. AA4. *J Am Chem Soc* 2011b;**3**:11434–7.

Shima J, Hesketh A, Okamoto S *et al.* Induction of actinorhodin production by *rpsL* (Encoding Ribosomal Protein S12) mutations that confer streptomycin resistance in *Streptomyces lividans* and *Streptomyces coelicolor* A3(2). *J Bacteriol* 1996;**178**:7276–84.

Shin CS, Kim HJ, Kim MJ *et al.* Morphological change and enhanced pigment production of *Monascus* when cocultured with *Saccharomyces cerevisiae* or *Aspergillus oryzae*. *Biotechnol Bioeng* 1998;**59**:576–81.

Shwab EK, Bok JW, Tribus M *et al.* Histone deacetylase activity regulates chemical diversity in *Aspergillus*. *Eukaryot Cell* 2007;**6**:1656–64.

Slattery M, Rajbhandari I, Wesson K. Competition-mediated antibiotic induction in the marine bacterium *Streptomyces tenjimariensis*. *Microb Ecol* 2001;**41**:90–6.

Sonnenbichler J, Dietrich J, Peipp H. Secondary fungal metabolites and their biological activities, V. Investigations concerning the induction of the biosynthesis of toxic secondary metabolites in basidiomycetes. *Biol Chem H-S* 1994;**375**:71–9.

Starosta AL, Lassak J, Jung K *et al.* The bacterial translation stress response. *FEMS Microbiol Rev* 2014;**38**:1172–201.

Sun J, Awakawa T, Noguchi H *et al.* Induced production of mycotoxins in an endophytic fungus from the medicinal plant *Datura stramonium* L. *Bioorg Med Chem Lett* 2012;**22**:6397–400.

Suzuki MM, Bird A. DNA methylation landscapes: provocative insights from epigenomics. *Nat Rev Genet* 2008;**9**:465–76.

Traxler MF, Seyedsayamdost MR, Clardy J *et al.* Interspecies modulation of bacterial development through iron competition and siderophore piracy. *Mol Microbiol* 2012;**86**:628–44.

Ueda K, Kawai S, Ogawa H *et al.* Wide distribution of interspecific stimulatory events on antibiotic production and sporulation among *Streptomyces* species. *J Antibiot* 2000;**53**:979–82.

VanderMolen KM, Darveaux BA, Chun W-L *et al.* Epigenetic manipulation of a filamentous fungus by the proteasome-inhibitor bortezomib induces the production of an additional secondary metabolite. *RSC Adv* 2014;**4**:18329–35.

Waksman SA. The role of antibiotics in nature. *Perspect Biol Med* 1961;**4**:271–87.

Williams RB, Henrikson JC, Hoover AR *et al.* Epigenetic remodeling of the fungal secondary metabolome. *Org Biomol Chem* 2008;**6**:1895–7.

Xu J, Tozawa Y, Lai C *et al.* A rifampicin resistance mutation in the *rpoB* gene confers ppGpp-independent antibiotic production in *Streptomyces coelicolor* A3(2). *Mol Genet Genomics* 2002;**268**:179–89.

Yamanaka K, Oikawa H, Ogawa HO *et al.* Desferrioxamine E produced by *Streptomyces griseus* stimulates growth and development of *Streptomyces tanashiensis*. *Microbiology* 2005;**151**:2899–905.

Yim G, Wang HH, Davies J. Antibiotics as signaling molecules. *Philis T R Soc B* 2007;**362**:1195–200.

Yu JH, Keller N. Regulation of secondary metabolism in filamentous fungi. *Annu Rev Phytopathol* 2005;**43**: 437–58.