J Ind Microbiol Biotechnol (2014) 41:415–424
DOI 10.1007/s10295-013-1387-y

REVIEW

SIMB
Society for Industrial Microbiology and Biotechnology

Activating secondary metabolism with stress and chemicals

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Received: 1 November 2013 / Accepted: 22 November 2013 / Published online: 11 December 2013

Abstract The available literature on the secondary or nonessential metabolites of the streptomycetes bacteria suggests that there may be poorly expressed or "cryptic" compounds that have yet to be identified and that may have significant medical utility. In addition, it is clear that there is a large and complex regulatory network that controls the production of these molecules in the laboratory and in nature. Two approaches that have been taken to manipulating the yields of secondary metabolites are the use of various stress responses and, more recently, the use of precision chemical probes. Here, we review the status of this work and outline the challenges and opportunities afforded by each of them.

Keywords *Streptomyces* · Secondary metabolism · Stress response · Chemical probe

Introduction

Secondary metabolites, also referred to as natural products [20, 82] or collectively as the parvome [21–23], are small, organic molecules that have diverse and often very potent biological activities. In most cases, they are dispensable for the viability of the producer, at least in the laboratory, but it is likely that they confer a significant selective advantage in nature. While many organisms produce secondary metabolites, the actinomycetes, including in particular the bacterial genus *Streptomyces,* are an especially rich source.

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The biological roles of many secondary metabolites are currently unknown. For example, it is unclear why *Streptomyces coelicolor* produces the light-harvesting molecule isorenieratene [70], a molecule that has been best characterized in the green sulphur bacteria [54]. It is also unclear why many bacterial cell walls and spores are decorated by pigmented secondary metabolites such as the gray-pigmented WhiE polyketide, also produced by *S. coelicolor* [18, 24]. Geosmin, the compound that confers the "earthy" smell to soil, is produced by many actinomycetes and myxobacteria, and again, its biological significance is a mystery [36]. In contrast, other secondary metabolites serve obvious and well-described roles in the producing organisms. For example, the iron-binding siderophores are a means for cells to scavenge iron from the environment [16, 35, 56, 57].

However, the secondary metabolites that are of greatest interest are those that have the capacity to damage other organisms or inhibit their growth [8, 25, 75]. While these molecules have properties that are similar to chemical signaling molecules [34, 69, 78], it is likely that in nature they serve to defend the producer from competing organisms. Indeed, most of the clinical antibiotics and antifungal drugs, as well as anticancer, antiparasitic drugs and compounds used to modulate the immune system, are derived in their natural forms or semi-synthetically from this source. The recent discovery of neuroactive polyketides in mollusk-associated bacteria from the Pacific Ocean further extends the range of biological activities associated with the parvome [51].

Secondary metabolites in current use were first discovered in small academic labs [67, 72, 73] while most of the compounds in use to day were discovered through giant screens of tens of thousands of strains conducted in the private sector [4, 5]. Most of this screening involved the

testing of culture supernatants for the capacity to inhibit the growth of common pathogenic microorganisms such as *Staphylococcus aureus* or *Mycobacterium tuberculosis.* This work, conducted between 1940 and 1980, led to the discovery of most of the familiar antibiotics in the present armamentarium including the tetracyclines, chloramphenicol, vancomycin, daptomycin and many others (Fig. 1a) [75]. Screens against fungi led to the discovery of compounds such as the echinocandins and polyenes (Fig. 1b) used to treat fungal infections [6], as well as rapamycin which is used to modulate the immune system [48, 71]. Screens against cancer cell lines led to the discovery of compounds such as doxorubicin [3, 27] (Fig. 1c), used in cancer chemotherapy.

The view in the 1990s was that the extensive screening conducted previously had yielded most of the chemical diversity that exists in nature and that finding new metabolites of interest might be excessively difficult [4]. However, the sequencing of streptomycete genomes demonstrated that, remarkably, every streptomycete genome has the genetic capacity to generate 20–40 distinct secondary metabolites, far more than had been predicted previously [7, 44, 59]. While we do not know what the majority of these compounds do, it is clear that there is a great deal of diversity that is worth investigating. The difficulty is that many of these gene clusters are expressed at low levels during growth in the laboratory. This has led to efforts aimed at activating these genes and identifying previously unreported molecules.

There have been several lines of research directed to this end. Successful approaches include the use of antibiotic resistant mutations [26, 39, 42], the use of metabolic signals such as *N*-acetylglucosamine [66], and the manipulation of pathway specific regulators [46] and pleiotropic regulators [55]. Much of this work is also described in this issue [58, 84] so we will not dwell on it here. Our concern is the use of chemical and stress responses including classical approaches such as ethanol and heat shock and more recent attempts to use specific chemical probes identified through compound screens or inhibitor modeling.

Blunt instruments: triggering secondary metabolism with stress

The streptomycetes and many other Actinobacteria have defined life cycles. Typically, growth commences with spore germination and the generation of filamentous cells called "substrate hyphae", which form a colony or substrate mycelium. After a few days, a second cell type, the aerial hyphae, grows up into the air to form an aerial mycelium. The cells in this fuzzy surface layer go on to generate pigmented spores to complete the life cycle [30–32, 77]. The production of some secondary metabolites takes place in a manner that correlates roughly with the onset of aerial mycelium formation. This includes two pigmented compounds produced by the well-characterized species *Streptomyces coelicolor*: the prodiginines (red) and actinorhodin (blue) (Fig. 2). Some of the mutations that block the formation of the aerial mycelium also block the production of these pigmented compounds indicating that the expression of some secondary metabolites is tightly linked to growth and the developmental process in this bacterium. Another example is the production of streptomycin by *Streptomyces griseus*, which is induced as part of the A-factor cascade along with the sporulation pathway. Mutations that block the production of the A-factor signaling molecule also prevent streptomycin production [37, 41, 60]. While this hardwiring of some secondary metabolism with the developmental cycle appears to be relatively common, it appears that many secondary metabolites are either not regulated this way or are produced at such low levels that their products cannot be detected or purified easily. Some investigators refer to these as the "cryptic" secondary metabolites.

There is considerable evidence that some stress responses can be used to trigger the expression of secondary metabolic genes: two that have been applied widely are heat shock and ethanol shock, both of which act through damage to the cell envelope and by driving the accumulation of misfolded or unfolded proteins [64, 68, 81].

The production of jadomycin B (Fig. 3) by *Streptomyces venezuelae* is a classic example of eliciting a secondary metabolite in response to stress. Jadomycin B belongs to the family of benzoxazolophenanthridine antibiotics that have potent antibiotic activity against Gram-positive pathogens such as *Staphylococcus aureus* and *Staphylococcus epidermidis* [45]. During normal culture of *S. venezuelae*, relatively little jadomycin is produced. Shifting the temperature from 27 to 42 °C, causes greatly elevated yields of jadomycin B, which peaks at 12 h following heat shock with titers increasing to as high as 25 μg/ml. Similarly, shocking cultures with 6 % ethanol, induces enhanced yields of jadomycin to as high as 30 μg/ml without requiring the elevation of culture temperature [28, 29]. Cultures of *S. venezuelae* that were not subjected to heat/ethanol shock and were maintained at 27 °C produced negligible amounts of jadomycin. The biosynthetic gene cluster for jadomycin production is regulated by two pathway-specific transcription factors: the transcriptional activator gene *jadR1* and the repressor *jadR2* in addition to several other, less well characterized regulatory genes [79, 80] (Fig. 4). JadR2 negatively regulates jadomycin production by direct repression of *jadR1*. Both ethanol shock and heat shock negate this repression.

To date, the best understood mechanism of heat-inducible gene expression in the streptomycetes involves the

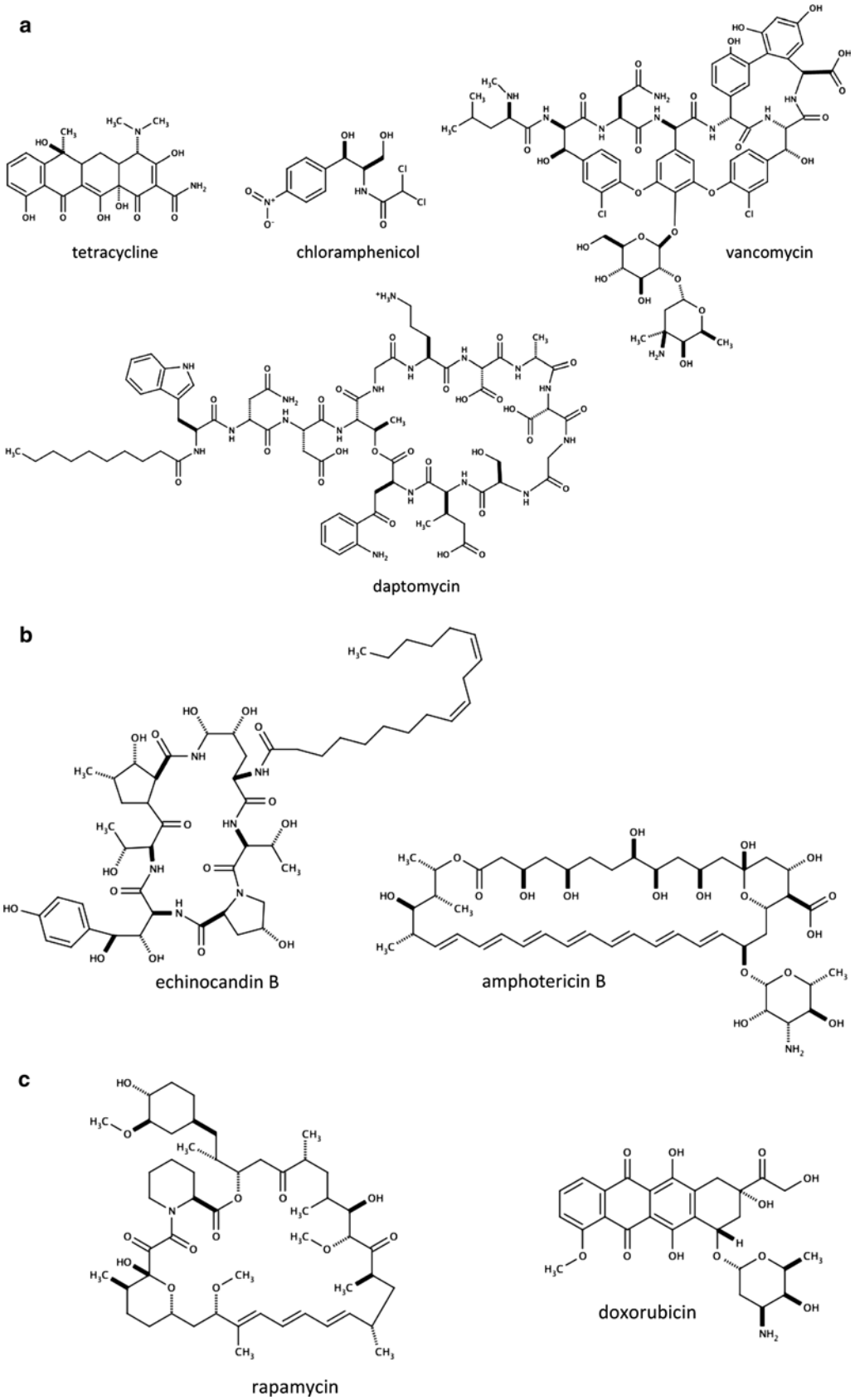


Fig. 1 Examples of commercially successful antibiotics (**a**), antifungals (**b**), and immune system modulating and antitumor compounds (**c**) discovered between 1950 and 1975

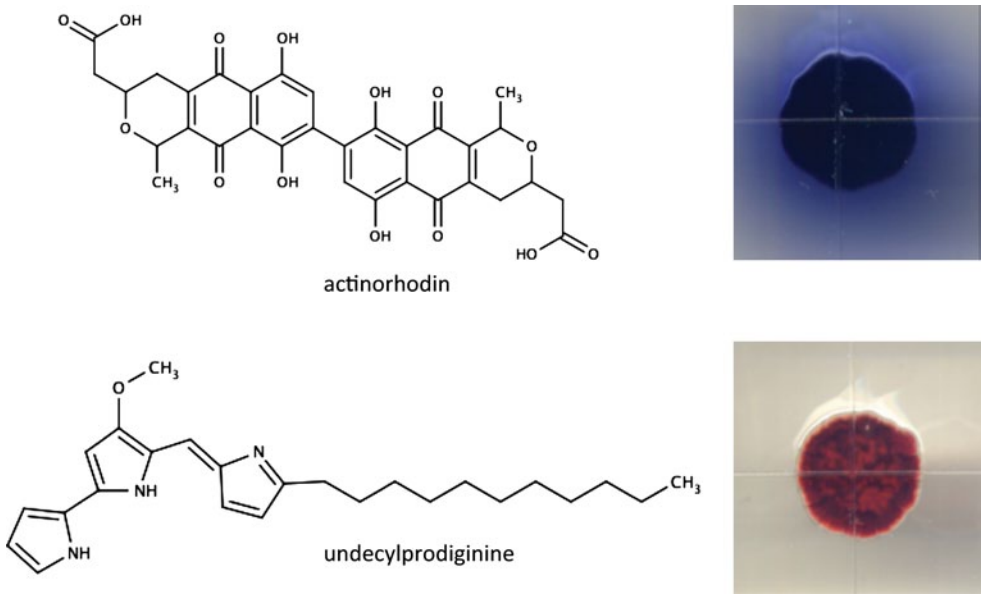


Fig. 2 Two pigmented compounds, actinorhodin and undecylprodiginine, produced by *S. coelicolor*

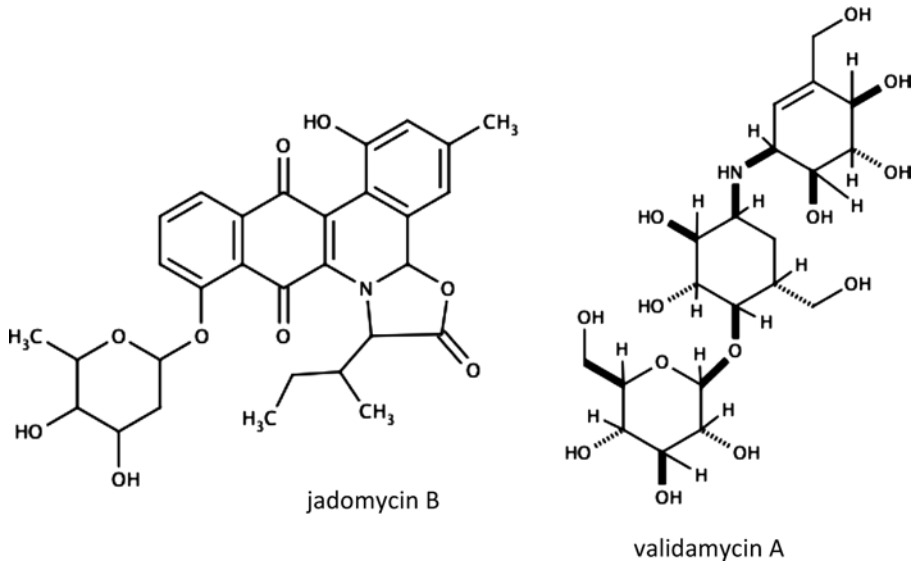


Fig. 3 Secondary metabolites induced by heat and/or ethanol shock. Jadomycin B produced by *S. venezuelae*. Validamycin A produced by *S. hygroscopicus*

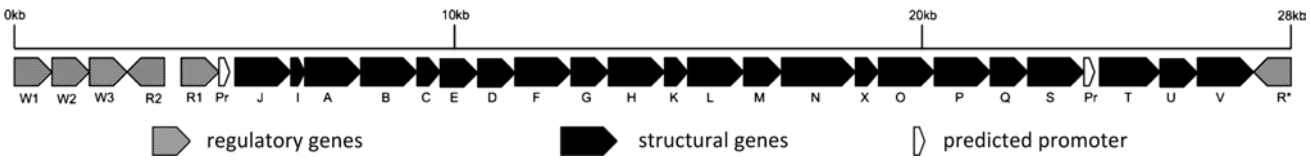


Fig. 4 Biosynthetic *jad* gene cluster

transcription factor HspR, which binds a palindromic sequence defined in *Streptomyces albus* by the consensus CTTGAGTNNNNNNNACTCAAG. This sequence, also known as the HspR-associated inverted repeat (HAIR), is found upstream of the coding sequence of the *dnaK grpE dnaJ hspR* operon—binding of HspR represses transcriptional initiation [68] (Fig. 5). It is believed that the chaperone protein DnaK binds HspR to serve as a co-repressor, stabilizing the binding of HspR to its recognition element [11–13]. In this scenario, mis-folded proteins that accumulate during heat shock would titrate DnaK away from HspR causing it to release the promoter sequence

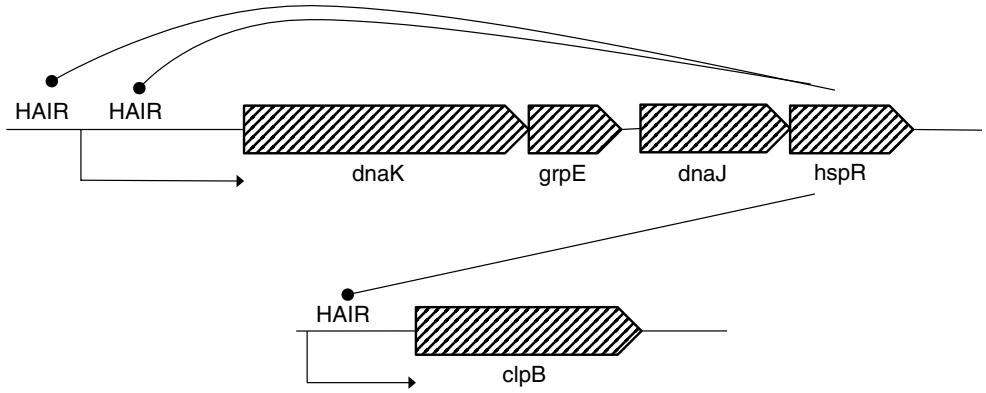


Fig. 5 HspR regulon of *Streptomyces albus*. HspR binds to HAIR (HspR-associated inverted repeat), as represented by the *filled circles*, serving as a repressor of the *dnaK* operon and the *clpB* gene

and permit transcriptional initiation. Other targets of HspR include *clpB*, *lon*, *rrnD*, and the gene for a Q/D tRNA [14]. While it is possible that the stress-induced accumulation of chaperone proteins and proteases contributes to the enhanced yields of jadomycin and other secondary metabolites, it is clear that this is not the full picture. There are no candidate binding sites for HspR in the jadomycin biosynthetic gene cluster and there is no obvious hypothesis for how these stress responses influence the known transcriptional regulators in the *jad* biosynthetic gene cluster.

Another compound that is produced in response to the heat/ethanol shock pathways is validamycin A (Fig. 3) by *Streptomyces hygroscopicus*. Validamycin A is an anti-fungal aminocyclitol antibiotic that is used as a primary control against sheath blight disease of rice and wheat plants [50]. Stressing producer cells with heat and/or ethanol can confer yields on the order of 13 g/l. Again, the mechanism by which the stress response elevates yields of validamycin A is not understood, however, it has been reported that heat and ethanol shock caused an increase in reactive oxygen species, which could also play a role [76, 83].

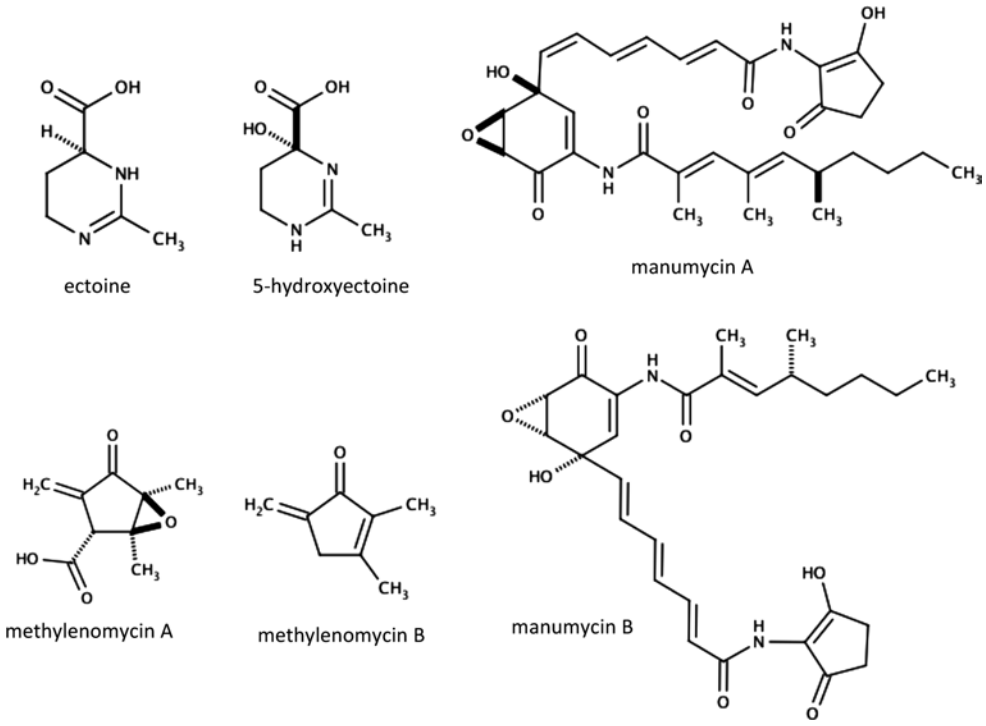
Altering a single parameter in the growth conditions and eliciting a stress response has been previously applied through the one strain many compounds (OSMAC) approach to explore the secondary metabolic potential of different strains [9]. Several other stress responses have been explored to this end in addition to ethanol and heat shock. *Streptomyces parvulus* yields new metabolites belonging to the manumycin family upon increasing the hydrostatic pressure during fermentation (Fig. 6) [9]. *S. coelicolor* produces the secondary metabolites ectoine and 5-hydroxyectoine in response to high salt and/or high temperature conditions (Fig. 6) [15]. Additionally, methylenomycin production by *S. coelicolor* can be triggered by either alanine growth-rate-limiting conditions and/or acidic pH shock (Fig. 6) [38]. Again, the mechanisms by which these environmental signals trigger secondary metabolism are poorly understood. We suggest that this area is ripe for further investigation using genome-scale technologies.

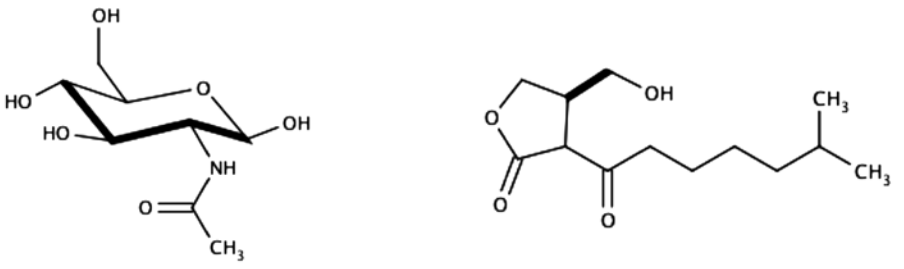
Precision tools: chemical elicitors of secondary metabolism

A large number of regulatory mechanisms impinge on secondary metabolism. Some of these respond to the availability of primary metabolites (e.g., nitrogen metabolism [74]), cell wall biosynthesis and homeostasis (*N*-acetyl glucosamine [66], peptidoglycan damaging antibiotics [39]), and other inputs [18, 52]. While there does not appear to be a universally conserved regulatory network for secondary metabolism, there are many shared regulatory genes. For example, pathway-specific regulators control the expression of many secondary metabolite pathway genes. The genes that encode these proteins are in turn under the control of more globally acting pleiotropic regulators. Indeed, it has recently been shown that the biosynthetic gene cluster for the phosphoglycolipid antibiotic moenomycin, which does not appear to have a pathway-specific regulator, is nevertheless under the control of three pleiotropic regulators that are highly conserved in many streptomycetes [53]. This suggests that some of the regulatory logic behind secondary metabolism is widely shared and that it should be possible to identify widely applicable methods for potentiating yields of cryptic secondary metabolites in many streptomycetes.

Classical genetics has played a vital role in the identification of many of the known secondary metabolism regulators [1, 60]. There has been steady progress in the understanding of these genes, however a great deal remains to be elucidated. We argue that new approaches are required to tease the diverse mechanisms apart and understand how they interact with each other and the biosynthetic genes. Our preferred direction, and a field that is in its infancy in

Fig. 6 Examples of secondary metabolites produced upon altering environmental conditions





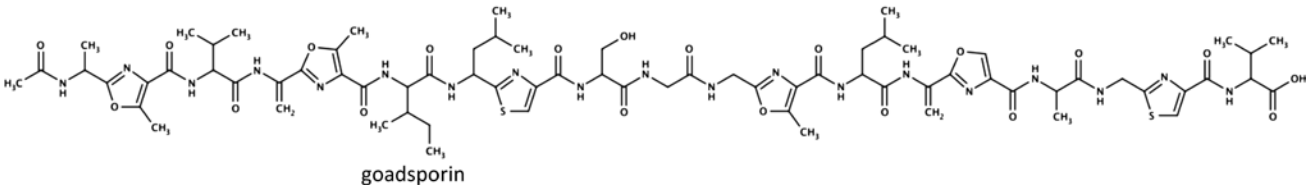


Fig. 7 Natural chemical probes of secondary metabolism

the streptomycetes, involves exploring the effects of chemical probes on the transcriptome and the parvome.

In addition to *N*-acetylglucosamine (Fig. 7) [66, 84], there have been several attempts to use chemical probes to induce the production of secondary metabolites. One early example involved a chemical substance designated goadsporin, a 19-amino-acid modified oligopeptide purified from *Streptomyces* sp. TP-A0584 [61, 62] (Fig. 7). Goadsporin was shown to stimulate the production of prodiginine antibiotics in *S. lividans*, and promoted pigment production and morphogenesis on 36 streptomycetes [61, 62].

Another class of naturally produced chemical probes is the autoregulators, including in particular the classic example of A-factor, a γ-butyrolactone that regulates the onset of streptomycin production in *S. griseus* (Fig. 7). Another secreted autoregulatory compound called PI factor, a 2,3-diamino-2,3-bis(hydroxymethyl)-1,4-butanediol, was isolated from *Streptomyces natalensis.* PI factor was active at very low concentrations and was shown to induce

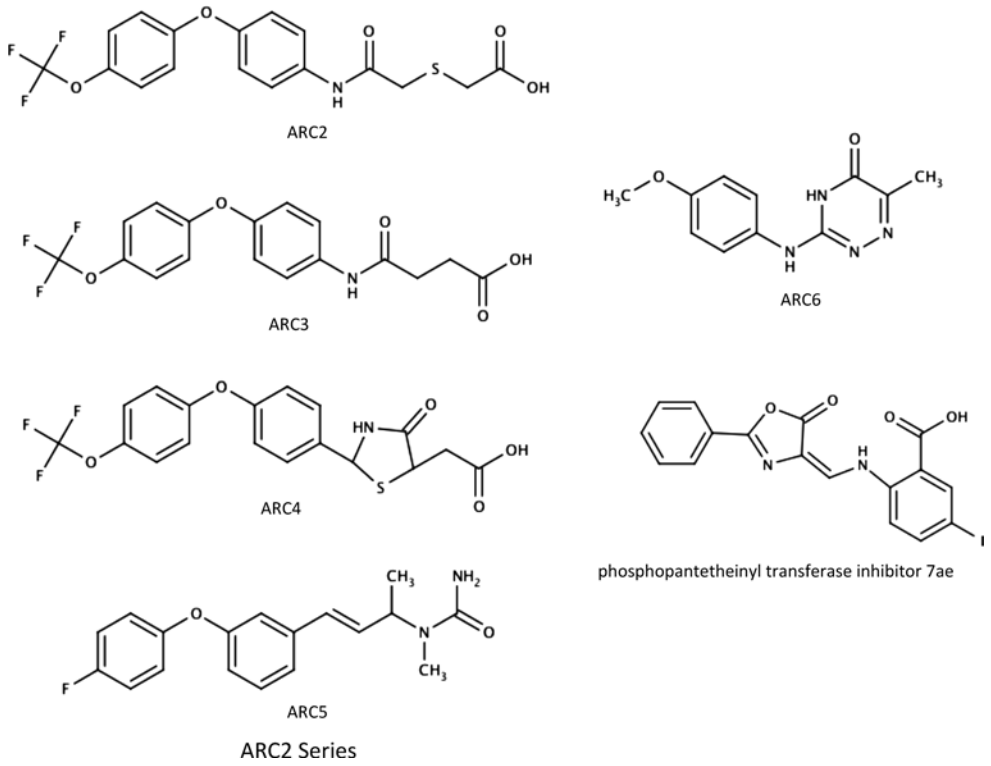


Fig. 8 Synthetic small-molecule probes of secondary metabolism

production of pimaricin in *S. natalensis*. Pimaricin, a glycosylated polyene, is important for antifungal therapy and shows promising antiviral activity [65]. More recently, a third class of hydroxymethylfuran signaling molecule was identified in *S. coelicolor* and shown to activate production of the plasmid encoded methylenomycin genes [17]. Synthetic analogues of these classes of signaling molecules would be rich terrain for identifying new chemical elicitors of secondary metabolism.

The first synthetic molecule shown to influence secondary metabolism was an inhibitor of phosphopantetheinyl transferase, an enzyme involved in activating the acyl carrier protein of fatty acid biosynthesis (Fig. 8). The compound, named 7ae, was synthesized based on the anthranilate 4*H*-oxazol-5-one pharmacophore [33]. Intriguingly, 7ae enhanced actinorhodin production when added to cultures of *S. coelicolor.* The mechanism of action for this compound is unknown.

The most comprehensive attempt to apply chemical genetics to secondary metabolism involved our screen of >30,000 compounds against *S. coelicolor* [19]. This resulted in the identification of 19 compounds that enhanced yields of the blue-pigmented compound actinorhodin. Four of these molecules, referred to as the ARC2 series (ARC2, ARC3, ARC4, ARC5) (Fig. 8), were structurally related to the synthetic antibiotic triclosan and appear to act by inhibiting the enoyl reductase FabI, thus implying that like 7ae, they target fatty acid metabolism. In this work, the link to fatty acid metabolism was revealed through the use of a triclosan resistant enoyl reductase, FabV, which interfered with the effect of the ARC2 series on secondary metabolism. The initial hypothesis advanced in this work was that by down-regulating fatty acid production, ARC2 causes a shunting of precursor molecules, in particular acetyl-CoA and malonyl-CoA, from fatty acid biosynthesis to secondary metabolism [19].

More recent work [2], however, suggested that the mechanism of action of ARC2 might be more complicated. Specifically, it was found that ARC2 did not reduce levels of fatty acids in cells, but rather that they caused an accumulation of greater levels of unsaturated fatty acids. Since there was no overall reduction in fatty acid levels in response to ARC2, it appeared less likely that a simple freeing up of precursors could account for the compound's action. The key may involve the shift in the class of fatty acids in ARC2-treated cells. Unsaturated fatty acids are normally scarce in bacterial cells and cell membrane—perhaps these molecules perturb the regulatory or biosynthetic machinery of secondary metabolism in some way? Indeed, in this work it was also found that another compound, ARC6, which is chemically unrelated to ARC2 and likely has a different molecular target, nevertheless also causes an

alteration in the fatty acid pools of cells. In this case, the result is an increase in branched chain fatty acids. At present, the actinorhodin-stimulating molecular mechanisms of these compounds are unknown, but it is striking that 7ae, ARC2, and ARC6 all have putative or demonstrated links to fatty acid metabolism.

Importantly, some of these probes appear to have potential use as widely active elicitors of secondary metabolism. In particular, ARC2 altered the secondary metabolite output of all of the >60 streptomycetes to which it has been applied so far. In all strains the yield of at least one compound [2, 19; Pimentel-Elardo et al., unpublished observations]. This includes enhanced yields of antibiotics, antifungals and, most importantly, a number of unknown compounds of unknown function. Indeed, the yield of at least one compound was elevated in each strain—in some there is evidence of reduced yields of selected compounds as well. This illustrates one important use of these chemical probes: they can be used to drive the unbiased production of poorly expressed secondary metabolites, which can then be purified and characterized.

What next?

Great strides have been made in our understanding of the molecular mechanisms governing the expression of the secondary metabolic genes. It's clear, however, that the links of the secondary metabolic genes to many of the best-characterized stress responses and chemical probes remain decidedly murky. Similarly, the mechanisms of action of the growing number of chemical probes of secondary metabolism (both synthetic and naturally occurring) are also poorly understood. There have already been a number of elegant genome-scale explorations of secondary metabolism and other aspects of the streptomcyetes life cycle [10, 40, 43, 47, 49]. We suggest that applying this technology to the stress responses and the chemical probes is the most direct path to a greatly enhanced understanding of the regulatory logic behind secondary metabolism.

One particularly informative approach might be to conduct a transcriptomic and metabolomics investigation of three well-characterized streptomycetes. For example, *S. coelicolor*, *S. griseus*, and *S. avermitilis*, each of which produce known secondary metabolites, also possess biosynthetic genes for many more that are unknown. Each strain could be subjected to one or more physiological stresses and/or one or more chemical probes of secondary metabolism. RNA could be extracted from each strain and small molecules extracted from the cognate culture supernatant. The changes in expressed transcripts and extracellular small molecules could then be explored using NG DNA sequencing and LC/MS, respectively. Given that most secondary metabolic genes have already been identified in these three species [7, 44, 59, 63], it should be possible to link induced molecules to induced gene clusters relatively easily.

An alternative approach would focus exclusively on the secondary metabolites themselves. For example, one stress or one chemical probe could be applied to cultures of a collection of freshly isolated streptomycetes. Compounds could be extracted from culture supernatants and each supernatant (±eliciting condition) could be subjected to LC/MS to identify induced compounds. Indeed, both approaches could similarly include the screening of culture extracts for induced antibacterial, antifungal or other biological activities.

The opportunity here is to develop a suite of technologies that could be used to induce sufficient yields of all of the secondary metabolites encoded in a given streptomycetes genome. Achieving this goal will require the elucidation of the molecular genetic mechanisms controlling the action of the existing chemical probes and of new chemical probes as they are identified. Integrating this information into the growing number of pathways linking biological inputs to secondary metabolism will make it possible to identify many of the cryptic secondary metabolites and investigate their biological effects in greater detail.

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