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REVIEW

Triggers and cues that activate antibiotic production by actinomycetes

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Abstract Actinomycetes are a rich source of natural products, and these mycelial bacteria produce the majority of the known antibiotics. The increasing difficulty to find new drugs via high-throughput screening has led to a decline in antibiotic research, while infectious diseases associated with multidrug resistance are spreading rapidly. Here we review new approaches and ideas that are currently being developed to increase our chances of finding novel antimicrobials, with focus on genetic, chemical, and ecological methods to elicit the expression of biosynthetic gene clusters. The genome sequencing revolution identified numerous gene clusters for natural products in actinomycetes, associated with a potentially huge reservoir of unknown molecules, and prioritizing them is a major challenge for in silico screening-based approaches. Some antibiotics are likely only expressed under very specific conditions, such as interaction with other microbes, which explains the renewed interest in soil and marine ecology. The identification of new gene clusters, as well as chemical elicitors and culturing conditions that activate their expression, should allow scientists to reinforce their efforts to find the necessary novel antimicrobial drugs.

Keywords Silent antibiotic · Elicitor · Soil ecology · Natural product · Genome mining · *Streptomyces*

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Introduction

The discovery of penicillin by Sir Alexander Fleming [42] opened up a completely new era of chemotherapy. The discovery of numerous antibiotics from primarily soil microorganisms and the near eradication of diseases such as tuberculosis led to the concept that infectious diseases may be something of the past [53]. However, the emergence of infectious diseases involving multidrug resistant (MDR) bacterial pathogens since the 1980s means that bacterial infections are still a major threat for human health. According to the World Health Organization (WHO), around 440,000 new cases of multidrug-resistant tuberculosis (MDR-TB) are found annually, causing more than 150,000 deaths. Extensively drug-resistant tuberculosis (XDR-TB) has now been reported in 64 countries to date [159]. The explosive increase in infections by pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecium* (VRE) and fluoroquinolone-resistant *Pseudomonas aeruginosa* is estimated to cause approximately 19,000 deaths per year in the US [70], and the most recent occurrence of pan-antibiotic-resistant infections pose the grave threat of completely untreatable infections [8].

Filamentous microorganisms (fungi and bacteria of the order of Actinomycetales) are the major source of secondary metabolites, producing some 90 % of all known antibiotics [15, 100]. Some two-thirds of all antibiotics are produced by actinomycetes, the majority of which by members of the genus *Streptomyces*. Until now, tens of thousands of natural antimicrobial products have been isolated from microbial sources, and still these likely represent only a tiny portion of the repertoire of bioactive compounds that can potentially be produced [15, 100]. Also, the microbial biodiversity of soil and marine

environments is enormous, with millions of fungi and bacteria likely to be present, of which we have seen only the tip of the iceberg [119].

The decline of high-throughput screening of antibiotics and silent gene clusters

As early as the 1990s, it was predicted that the future of antibiotic discovery might not lie in high-throughput screening (HTS; [71]) or combinatorial chemistry [97]. Perhaps the best known example is the HTS effort conducted by GlaxoSmithKline, where millions of compounds were screened in many HTS campaigns, with only marginal success [117]. Underlying causes for the lack of success include the fact that many of the essential targets in the bacterial cell are not "druggable", that the molecules identified by HTS do not always have the ideal drug properties as defined in Lipinski's rule-of-five [78], and that molecules in compound libraries are biased for use in pharmacology and do not have the molecular complexity of naturally occurring secondary metabolites.

Antibiotics not yet discovered have been estimated to be produced at frequencies of less than one per million in fermentation broths from randomly chosen actinomycetes [10, 12]. The situation is often hindered by the fact that often actinomycetes produce high levels of several antibiotics, which will obscure the production of less well expressed or less bioactive antibiotics. How can we stand a chance to find anything novel when BigPharma fails to find them in their comprehensive screening efforts? For one, actinomycetes are soil or marine bacteria, and one approach most likely lies in the direction of ecology. In other words, if we understand the temporal and conditional cues that activate antibiotic production in situ, this can then be applied to improve screening efforts. A second aspect that is now available is the information derived from whole genome sequencing and the connected genomics technologies. Sequencing the genomes of actinomycetes established the presence of many more biosynthetic clusters for secondary metabolites than originally anticipated. For example, it has been known for decades from the pioneering work of David Hopwood and many of his colleagues that the model actinomycete *Streptomyces coelicolor* produces four antibiotics, namely actinorhodin (Act), undecylprodigiosin (Red), calcium-dependent antibiotic (Cda), and the plasmid-encoded methylenomycin (Mmy) [51, 53]. Still, despite 50 years of intensive research, it came as a complete surprise when the *S. coelicolor* genome sequence [14] revealed the presence of many previously unidentified biosynthetic gene clusters [25, 53], including one for a likely antibiotic called cryptic polyketide (Cpk; [116]). It rapidly became clear that other actinomycetes also have extensive arsenals of secondary metabolites [59, 109, 111, 112, 147]. It therefore appears that the potential of these organisms for novel drug production is much larger than originally anticipated. This has led to extensive research into the applied genomics relating to what is generally referred to as cryptic, silent, or sleeping antibiotics (reviewed in [46, 94, 99, 152, 169]).

Therefore, there are likely many yet-unidentified compounds out there, which were missed either because the gene clusters that specify them are not expressed at sufficiently high levels or because the compounds have lower specific activity than the readily screenable antibiotics and may require modification to become more active. Here we review in particular global approaches for antibiotic mining, with the activation of poorly expressed antibiotic biosynthetic gene clusters in mind.

Regulation of antibiotic production

The regulation of antibiotic production involves multiple regulatory cascades and networks. Knowledge on the regulatory genes can be applied in approaches to activate antibiotic production but is also very useful in terms of localizing biosynthetic gene clusters (see below). Although the number and variety of genes involved vary from species to species, certain features are common. Global regulators almost by definition have a wide-ranging impact on global transcription patterns, but also "cluster-situated regulators" (CSRs) may have a broader impact than only on the cluster they are associated with [58]. Genes involved in antibiotic production are generally organized in biosynthetic gene clusters, consisting of several transcription units. Besides the obvious advantage of coordinated control of biosynthesis, export, and resistance, the additional evolutionary driving force behind such linkage is most likely an ecological one, as it allows the transfer of complete gene clusters during genetic exchange in the habitat.

The *act* gene cluster as model system

Arguably the best studied antibiotic gene cluster is *act* in *S. coelicolor*, for the type-II PKS actinorhodin (Act). This genetic system is a beautiful illustration of the possible complexity of the regulatory networks involved in the control of antibiotic production, and we highlight common features to illustrate the relevant concepts, in particular because most pleiotropic regulators have an effect on Act production in *S. coelicolor*. For more extensive overviews of the control of antibiotic production, we refer to reviews elsewhere [16, 79, 151]. Several general themes related to the control of *act* production are highly relevant for approaches to wake up sleeping antibiotics, and are worked out in more detail in the following sections of this review.

The *act* gene cluster consists of around 20 genes, organized in several transcription units [82]. ActII-ORF4 is the cluster-situated and pathway-specific regulator of the *act* gene cluster that binds to target sequences of the *act* promoters, with an N-terminal helix-turn-helix DNA binding domain and a C-terminal transcriptional activation domain [62]. ActII-ORF4 is a member of a family of pathway-specific activator proteins termed *Streptomyces* antibiotic regulatory proteins or SARPs [160]. SARP regulators are typically expressed in a growth phase-dependent manner and at a high level [16], and there appears to be little or no control downstream. This is exemplified by promoter probing experiments using *redD*—the pathway-specific activator gene for the *red* cluster—as a reporter; which demonstrated that both timing and level of expression of the *red* cluster is directly proportional to the expression of RedD, even in early vegetative mycelia or in aerial hyphae, where the cluster is normally not expressed [154].

Many pleiotropic regulators characterized so far are required only under specific environmental conditions [17], and several of these control *act* gene expression. DasR is a GntR-family regulator that controls among others amino sugar metabolism and transport and the chitinolytic system [29, 30, 124, 140]. DasR is a highly pleiotropic regulator, and in a recent environmental study, microarray data on the *dasR* null mutant in soil-grown cultures in the presence of chitin revealed some 700 genes that were differentially expressed [98]. DasR connects the control of primary and secondary metabolism by directly controlling the transcription of *act*II-ORF4, the pathway-specific activator gene for actinorhodin biosynthesis (Fig. 1), and *redZ*, a response regulator required for undecylprodigiosin production. The DasR regulon and its use as target for global approaches to induce antibiotic production is discussed in detail below.

AtrA is a TetR-family protein that is required for the transcription of *actII*-ORF4 [148], and in turn responds to the level of phosphate as it is repressed by the PhoRP system [130]. Further complexity is offered by Rok7B7, a member of the ROK family of proteins, which are

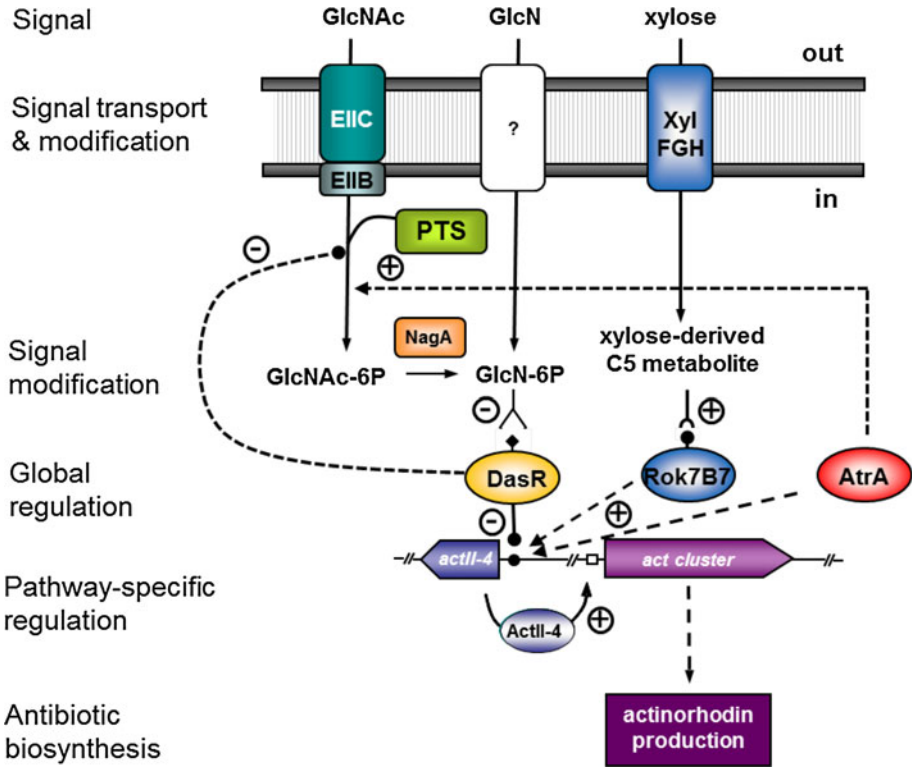


Fig. 1 Pleiotropic and nutrient-mediated control of actinorhodin production in *S. coelicolor*. *N*-acetylglucosamine (GlcNAc) enters the cell and is subsequently phosphorylated via the GlcNAc-specific phosphoenolpyruvate-dependent phosphotransferase system (PTS), composed of intracellular PTS proteins EI, HPr, and EIIA, and the GlcNAc-specific components EIIB (NagE2) and EIIC (NagF). Phosphoenolpyruvate (PEP) is the phosphodonor. *N*-acetylglucosamine-6-phosphate (GlcNAc-6P) is then deacetylated by GlcN-6P deacetylase NagA to glucosamine-6-phosphate (GlcN-6P), the effector molecule that inhibits DasR DNA-binding. This results in derepression of *act*II-ORF4, the pathway-specific transcriptional activator gene for the actinorhodin biosynthetic gene cluster. This represents a complete signaling cascade from extracellular nutrients to the activation of actinorhodin production. The global regulators AtrA and Rok7B7 have opposite activities to DasR, with AtrA and Rok7B7 both activating actinorhodin production, and at least AtrA also activating GlcNAc transport, thereby antagonizing DasR. Rok7B7 is likely activated by a xylose-derived C5 sugar transported via the ABC transporter XylFGH. For details and references, see the text. The effect of GlcNAc on antibiotic production is shown in Fig. 2

predominantly sugar regulatory proteins and sugar kinases, including glucose kinase [146]. Rok7B7 pleiotropically affects primary and secondary metabolism, and is required for actinorhodin production [139]. Introduction of an ortholog of *rok7B7* called *rep*, obtained from a metagenomic library, appeared to be an effective way of activating antibiotic production in *S. coelicolor* [90]. Recent evidence suggests that Rok7B7 may be activated by a derivative of the C5 sugar xylose [139]. The activity of DasR, Rok7B7, and perhaps also AtrA is subject to nutrient control at the posttranslational level, and the metabolic status of the cell will therefore largely determine their contributions to the control of antibiotic production.

Other pleiotropic antibiotic regulators involved in the control of *actII*-ORF4 are AfsR and PhoP. AfsR contains an N-terminal SARP domain and is conditionally required for Act and Red production [43]. AfsR binds to the promoter of the downstream located *afsS* (also called *afsR2*) and activates its transcription [75]. While the precise function of *afsS* is unclear, it activates antibiotic production in many streptomycetes, and is therefore an attractive target for the activation of antibiotic production. PhoP, which represses actinorhodin production in response to phosphate, probably acts indirectly, and perhaps by repressing *afsS*. The role of PhoP in the control of antibiotic production is discussed in more detail in the next section.

Interestingly, deletion of the gene for *Streptomyces* integration host factor (sIHF) also effects enhanced actinorhodin production [166]. IHF assists in cell processes that require higher-order protein complexes, e.g., DNA replication, transcriptional regulation, and site-specific recombination [44]. Again, the effect of sIHF on antibiotic production is most likely direct, as EMSAs showed direct binding to the *redD* promoter region [166]. Finally, the two-component system DraR-K controls antibiotic biosynthesis in *S. coelicolor* in response to high concentrations of nitrogen [168]. Phospho-DraR enhances the production of Act but represses yellow pigmented type I polyketide (yCPK) biosynthesis via direct control of the pathway-specific activator genes *actII*-ORF4 and *cpkO*, respectively, while repression of Red biosynthesis is indirect [168]. Besides the selection of regulatory proteins mentioned above, a surprisingly large number of other genes affect the expression of *actII-ORF4* [79, 151]. This highlights the amazing regulatory complexity for a gene cluster that apparently specifies a compound with weak antibiotic activity, suggesting that *actII*-ORF4 and/or the *act* cluster may have a more important physiological role than currently anticipated.

Genetic control in response to carbon, nitrogen, and phosphorous

Most secondary metabolites are produced in a growth phase-dependent manner, typically during the onset of development, corresponding to transition phase and early stationary phase in submerged cultures. Nutrient composition and concentration within media not only affects growth rate but also influences complex changes in global gene regulation, reflecting the range of conditions that trigger the production of different antimicrobials in nature [16, 128, 151].

Carbon sources have a major impact on the expression of biosynthetic genes and morphological development of microorganisms. Carbon catabolite repression (CCR) occurs when media contains mixtures of rapidly and slowly used carbon sources and is a regulatory mechanism commonly observed in bacteria [22, 45, 145]. After exhausting the preferred carbon source, bacteria turn to the "second-best" carbon source, and this often correlates temporally with the onset of antibiotic production, which is associated with growth cessation [36]. Glucose is a preferred carbon source for many actinomycetes and effects global repression of antibiotic production [37, 118], which is why in industrial fermentations, polysaccharides (e.g., starch), oligosaccharides (e.g., lactose) and oils (e.g., soybean oil, methyloleate) are commonly used.

The central protein that controls CCR in streptomycetes is glucose kinase (Glk), with deletion of the gene for glucose kinase resulting in global derepression of carbon utilization [5, 6]. Glk expression is constitutive, and its activation takes place posttranslationally in glucose-grown cultures, but not when cultures are grown on non-repressing sugars [149]. This suggests that interfering with the activation of the CCR activity of Glk, while leaving glycolysis unchanged, should enable antibiotic production in glucose-grown cultures, which would be a major advantage for industrial fermentations. To address this, a comprehensive quantitative proteome analysis was performed on cultures of *S. coelicolor* and its *glkA* mutant grown in minimal media with mannitol or fructose and with or without additional glucose, which revealed the response of nearly all enzymes in central metabolism and most antibiotic-related pathways. This surprisingly showed that while CCR and inducer exclusion of the majority of the primary and secondary metabolic pathways was mediated in a Glk-dependent manner (as expected), glucose repression of the biosynthesis of the γ-butyrolactone Scb1 and the responsive *cpk* gene cluster for the cryptic polyketide Cpk is independent of Glk [47]. Other cryptic pathways could perhaps also be controlled in an entirely different way, providing a possible new lead for the activation of poorly expressed antibiotics.

High concentrations of nitrogen sources such as ammonium or amino acids also suppress the biosynthesis of secondary metabolites [1, 86]. Complex fermentation media therefore include proteins as nitrogen source and defined media slowly assimilated amino acids, so as to

ensure optimal antibiotic production. For example, streptomycin production by *Streptomyces griseus* is favored by growth in both soybean meal and proline in combination with low concentrations of additional ammonium salts [39, 162]. Production of aminoglycoside antibiotics was also repressed by ammonium salts, whereas nitrate and certain amino acids stimulated their production [132].

Biosynthesis of antibiotics and other secondary metabolites is transcriptionally repressed by easily utilized phosphate sources [85]. The depletion of phosphate in the environment triggers the biosynthesis of secondary metabolites but represses growth [86]. Generally, phosphate concentrations above 0.5 mM stimulate growth at the expense of secondary metabolism, while, conversely, below this threshold the production of secondary metabolites is favored, including antibiotic production [156]. This activation of antibiotic production is among others governed via autoregulatory signals [54, 101, 164]. A major system for the global control of gene expression in response to the phosphate concentration is the two-component regulatory system PhoRP [136]. PhoRP is widespread in prokaryotes and is involved in the control of antibiotic production in among others *S. coelicolor* and *Streptomyces lividans* (Act, Red; [129, 135]), *S. griseus* (candicidin; [87]), *S. natalensis* (pimaricin; [95]) and in *Streptomyces rimosus* (oxytetracycline; [92]), although phosphate-mediated repression is a general phenomenon [88].

Sensory histidine kinase PhoR is prevented from phosphorylating its cognate response regulator PhoP via its interaction with the high-affinity phosphate transport system Pst, which in turn is activated by PhoR. Thus, only little phosphate transporter is produced when sufficient phosphate is available. However, during phosphate limitation, PhoR is released and phosphorylates PhoP, resulting in enhanced phosphate transport and utilization [85, 136]. Recent global analysis of PhoP binding sites by immunoprecipitated genomic DNA hybridized to DNA microarrays (ChIP-chip) showed that besides controlling the phosphate regulon, PhoP also transiently shuts down central metabolic pathways [3]. PhoP directly controls the pathway-specific regulatory gene *cdaR* for Cda production in *S. coelicolor*, but control of actinorhodin and undecylprodigiosin production is most likely indirect, as there are no obvious PhoP-binding sites in the promoter regions of the pathway-specific regulatory genes *actII*-ORF4 and *redD/redZ*, respectively, and PhoP failed to bind to the promoters [85]. However, PhoP may affect these antibiotics indirectly via the control of *afsS* [130].

In addition, PhoP plays a major role in the cross-talk between N- and P-metabolism, via the repression of *glnR*, for the global nitrogen regulator GlnR [89, 126]. Therefore, PhoP takes up a central role in the junction between primary and secondary metabolism, and targeting this system is an attractive approach to pleiotropically affect antibiotic production.

Genetic tools for the activation of antibiotic production

Enhanced expression of SARP regulators is an effective yet strain-specific approach for the overexpression of the gene clusters they control. However, the associated gene clusters are often "household" antibiotics, i.e., they are expressed under most growth conditions, typically during the transition from late exponential to stationary growth [16]. This suggests that most antibiotics specified by SARP-controlled gene clusters will have been identified in HTS screening efforts by BigPharma.

Recently, a new class of regulatory genes was identified, which encode proteins with similarity to LAL (Large ATP binding regulators of the LuxR family) proteins [72]. Constitutive expression of a pathway-specific LAL regulator as CSR for a giant (150 kb) type-I modular polyketide synthase (PKS) gene cluster in *Streptomyces ambofaciens*, resulted in the production of a number of novel 51-membered glycosylated macrolides, named stambomycins A–D [72]. Since genome sequencing identified LAL regulators that are associated with several yet unidentified gene clusters, constitutive expression of such CSRs represents a promising new approach for natural product discovery.

The enhanced expression of *afsS* appears to be an effective and generally applicable way of activating antibiotic production. Its enhanced expression stimulates antibiotic production in among others *S. coelicolor* [91], *S. lividans* [157], *S. avermitilis* [74], and *S. noursei* [131]. The overexpression of AfsR also increased antibiotic production, for example in *S. coelicolor* [43], *S. peucetius* [115], and *S. venezuelae* [81], which is perhaps mediated via activation of *afsS* [75]. The effect of the *afsS* orthologue *ssmA* on nistatin production by *S. noursei* suggests that perhaps *afsRS* act in a carbon source-dependent manner [131].

Ribosome engineering

An effective and very promising way to activate antibiotic production is manipulation of the strains via so-called "ribosome-engineering", developed by Kozo Ochi and colleagues (recently reviewed in [106, 108]). Ribosome engineering is a method that uses sub-lethal concentrations of antibiotics that target either the ribosome itself or RNA polymerase (RNAP). Drug-resistant mutants enforced by rifampicin have mutations in *rpoB* (for the β-subunit of the RNAP), while those induced by streptomycin carry mutations in *rpsL* for ribosomal protein S12 or also in *rsmG* for

a 16S rRNA methyltransferase, which gives lower resistance. Combinations of mutations in *rpoB*, *rpsL*, or *rsmG* typically leads to further enhancement of antibiotic production [106, 142, 144]. The ribosome engineering technology proved to be successful in the activation of Act production in *S. coelicolor* and *S. lividans*, but was also successful in triggering antibiotic production in less well-studied actinomycetes, such as the production for novel piperidamycins by *S. mauvecolor* triggered by a number of different *rpoB* or *rpsL* mutations [56]. The wide application is further demonstrated by the fact that production of the normally silent amino sugar antibiotic 3,3′-neotrehalosadiamine (NTD) by the firmicute *Bacillus subtilis* was activated by an *rpoB* mutation [61]. Inducing resistance to several other antibiotics such as gentamicin, erythromycin, and capreomycin also activated antibiotic production, and resistance to these structurally diverse antibiotics typically relates to deletion or expression of an rRNA methyltransferase [63, 80, 134]. A major advantage from the application point of view is that introducing antibiotic resistant mutations can be done by a straightforward selection procedure, rather than by introducing specific mutations, and the technology therefore finds wide application [2, 13, 106, 120, 155]. For further details, we refer to the review by Ochi and colleagues elsewhere in this issue.

Ecological considerations and co-cultivation

If we are to activate silent antibiotic gene clusters, then understanding of their biological role is of major importance. Streptomycetes grow as a branched multicellular network of hyphae—the vegetative or substrate mycelium—and reproduce through spores that are formed by a specialized aerial mycelium. The onset of development is triggered by stress conditions such as drought or famine. A proportion of the vegetative mycelium lyses following a process of programmed cell death [83], which releases nutrients that presumably form the building blocks for the sporulation process. For a detailed description of the control of morphological differentiation of streptomycetes, we refer to excellent reviews elsewhere [27, 41, 52]. It is likely that the release of nutrients in an otherwise depleted environment attracts competing microbes, and it is logical to perceive the production of antibiotics as a defense mechanism. Indeed, many antibiotics are produced at a time correlating to the onset of development [16, 17, 151].

A major issue connected to existing strain collections is that they are just that, collections, i.e., the microorganisms have been taken out of their ecological context, and the strains are typically screened individually. Inevitably, in nature, many antibiotics will only be produced after receipt of specific signals, such as from the environment (stress) or from surrounding microbes (symbionts or competitors). Novel molecular ecological methods should aid us in understanding and identifying the triggers that activate the production of antibiotics in nature, which explains the rapidly growing interest in soil and marine ecology related to the production of antibiotics in the natural habitat. The original view that antibiotics are purely antagonistic, acting in nature as they do in the clinic, has recently been questioned [77, 121, 127]. First, the concentrations of antibiotics in the soil are argued to be too low to be efficacious. Second, sub-inhibitory concentrations of antibiotics induce novel phenotypic and genetic responses in exposed organisms, including increased biofilm formation and expression of virulence genes. Thus antibiotics may also act as "collective regulators of the homeostasis of microbial communities", in others words act as signals or cues rather than weapons [77, 121]. Antibiotics acting as signals enable symbiotic relationships between different organisms, each benefiting from either nutrition or protection. The way microbes influence each other has recently been beautifully displayed by Pieter Dorrestein and colleagues, who used imaging mass spectrometry to visualize secondary metabolites and signaling molecules produced by microbes grown in close proximity [68, 158, 167]. The power of this technology is that—in particular with increasing resolution—it may directly identify new chemical elicitors that activate antibiotic production by actinomycetes.

Co-cultivation of different bacterial species was applied successfully to activate the expression of novel antibiotics. The novel antimicrobial alchivemycin A was produced via the co-cultivation of *Tsukamurella pulmonis* TP-B0596, a mycolic acid-containing bacterium, together with *Streptomyces endus* S-522. It was proposed that mycolic acid located in the outer cell layer of *Tsukamurella* induced secondary metabolism in *Streptomyces* [113]. A competition-based adaptive assay has recently been developed, encouraging the evolution of an organism to produce antimicrobials via serial co-cultivations with a target pathogenic bacterium such as MRSA. Results revealed the activation of the anti-staphylococcal agent holomycin when *Streptomyces clavuligerus* was co-cultured with MRSA for consecutive passages until significant bioactivity was elicited [26]. While perhaps not feasible in larger screening efforts, such pair-wise interactions may identify important cues and triggers for poorly expressed antibiotics in actinomycetes, which may find wider application.

Chemical elicitors of antibiotic production

With the promise of finding novel antibiotics, the exploration of elicitors able to activate the expression of silent antibiotic biosynthetic gene clusters has begun, aimed at

optimal exploitation of the seemingly enormous potential. This section highlights some of the recent advances in this promising line of research.

N-acetylglucosamine and the DasR regulon

A signaling cascade from an extracellular nutrient to the activation of antibiotic production was elucidated in *S. coelicolor*, which is based on the derepression of the regulon of the GntR-family regulator DasR. Higher concentrations of the cell-wall component *N*-acetylglucosamine (GlcNAc; 5–10 mM) trigger development and antibiotic production under poor growth conditions ("famine"), while they activate development under rich ("feast") conditions (Fig. 2a; [125]). The final receptor of the GlcNAc-derived signal is DasR. GlcNAc is imported via the PEP-dependent phosphotransferase system PTS [102, 103], and subsequently metabolized to glucosamine-6-phosphate (GlcN-6P), an important starter molecule for cell-wall biosynthesis (Fig. 1). GlcN-6P is a ligand for DasR by binding to its effector binding site and thereby reducing the protein's affinity for DNA [124]. This is not unexpected, as GlcN-6P stands at the crossroads of $(GlcNAc)_n$ degradation, GlcNAc transport, and intracellular metabolism, glycolysis, nitrogen and lipid metabolism, as well as peptidoglycan synthesis [4, 84], and many genes of these pathways are subject to control by DasR. A complete signaling pathway was established from import of a signal (GlcNAc; first step) to the activation of pathway-specific regulatory genes (*actII-ORF4*, *redZ*; final step), see Fig. 1. Interestingly, AtrA appears to counteract DasR, by having opposite actions on the start and end of this signaling pathway. While DasR represses transcription of the genes for the GlcNAc-specific transporter NagE2 (signal import) and for ActII-ORF4 (antibiotic activation), both of these genes are transcriptionally activated by AtrA [103].

The transcription of all known chromosomally encoded antibiotic biosynthetic clusters of *S. coelicolor* (*act, cda, red*, and the "cryptic" *cpk* cluster) is enhanced in *dasR* mutants [125], while DasR also controls siderophore production [32]. Therefore, manipulating the activity of DasR should potentially allow triggering the expression of antibiotics. Indeed, growth of *S. coelicolor* on minimal media agar plates containing only GlcNAc as the carbon source accelerated development and enhanced antibiotic production, and this was also observed for a number of other actinomycetes [125, 152]. An example is presented in Fig. 2c, which shows the effect of GlcNAc on antibiotic production by six streptomycetes. Interestingly, the *cpk* cluster for the cryptic type I polyketide synthase Cpk is also induced by the addition of *N*-acetylglucosamine [125]. This provides one example of novel approaches that may be employed to boost the potential of novel screening procedures. Alternatively, creating mutants in the *nag* metabolic genes disturbed GlcNAc metabolism in such a way that metabolic intermediates accumulated intracellularly, resulting in increased antibiotic production [141].

It should be noted, however, that GlcNAc and glutamate are also important carbon and nitrogen sources for streptomycetes, and glutamate is preferred over glucose by *S. coelicolor* [102, 150]. The fact that GlcNAc and its direct metabolic derivatives promote growth may explain why the compound suppresses antibiotic production in a number of actinomycetes, even though it activates the production in others.

Chemical elicitors that modulate fatty acid biosynthesis

Besides GlcNAc, other molecules that result from macromolecule recycling should also be considered as elicitors, such as nucleotides from DNA and RNA, oligopeptides and amino acids from proteins, sugars from polysaccharides (including extracellular matrix EPS and LPS) and fatty acids from lipids. Interestingly, a recent screen by Justin Nodwell and colleagues of a chemical library of around 30,000 small molecules for compounds that can act as elicitors of antibiotic production, revealed a family of molecules that act by modulating fatty acid biosynthesis, referred to as antibiotic remodeling compounds (ARCs; [33]), which show similarity to the structure of the furan-like antibiotic triclosan (Fig. 3). Of the ARCs, ARC2 was the most active one. It acts via inhibition of the enoyl-acyl carrier protein reductase FabI, which is a key enzyme of type II fatty acid biosynthesis. Secondary metabolism and fatty acid biosynthesis compete for the common substrate acetyl-CoA, and ARC2 may act via the partial inhibition of FabI, thus allowing a preferential flow of acetyl-CoA to antibiotic production [33, 107].

However, besides interfering with fatty acid metabolism, triclosan is also known to affect quorum sensing and auto-induction mechanisms [38], which mediate cell–cell communication. Quorum sensing-regulated genes were among the most strongly downregulated genes in triclosan-treated *Pseudomonas aeruginosa* cells [28]. In streptomycetes, quorum sensing-like communication is mediated by γ-butyrolactones, and these play a role in the control of antibiotic production (see next section), and triclosan might also act by interfering with this system.

Extracellular signaling molecules

Microbial, hormone-like, small diffusible molecules known as γ-butyrolactones play a role in the communication between actinomycetes in the soil, controlling development and antibiotic production (recently reviewed in [57, 133, 161]. The best known example is A-factor (Fig. 3), a

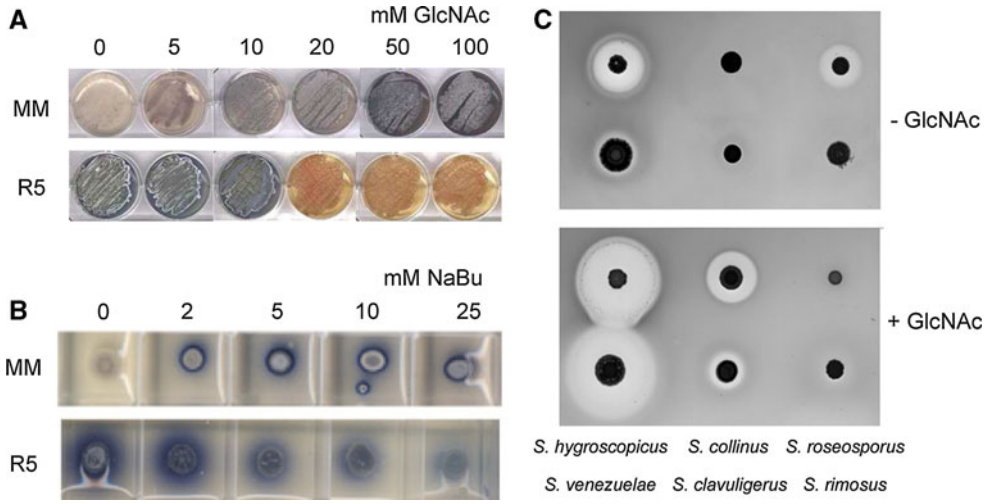
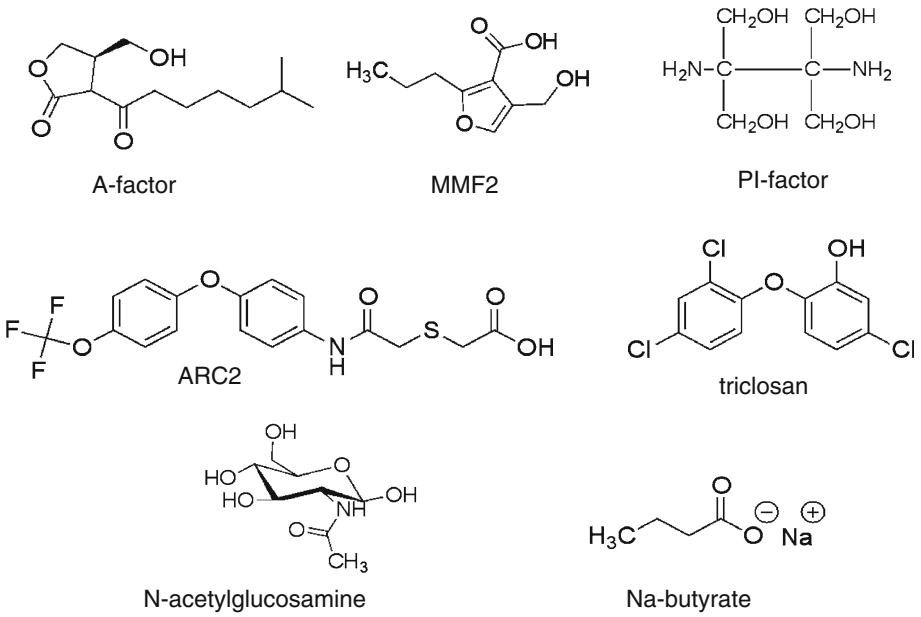


Fig. 2 Medium-dependent activation of antibiotic production by elicitors. Increasing concentrations of GlcNAc (**a**) or sodium butyrate (NaBu; **b**) repress antibiotic production and development under rich growth conditions (R5 agar plates; "feast") and activate developmental processes in cultures grown under poor conditions (MM agar plates; "famine"). GlcNAc acts by interfering with the activity of DasR, while NaBu is known to target histone deacetylase (HDAC). The *blue pigment* is the polyketide antibiotic actinorhodin, spores are *grey-pigmented*. **c** Effect of GlcNAc on antibiotic production by six selected streptomycetes. The strains were grown on minimal medium agar plates with mannitol (1 % w/v) or *N*-acetylglucosamine (25 mM) as the sole carbon source. *Bacillus subtilis* was used as indicator strain. Halos correspond to antibiotics produced by the streptomycetes. Note that *N*-acetylglucosamine inhibits antibiotic production by *S. roseosporus*. Fig. 2a, c based on [125], Fig. 2b adapted with permission from [96]

Fig. 3 Chemical elicitors of antibiotic production. Chemical structures are presented for known elicitors of antibiotic production and related molecules. For details, we refer to the relevant sections on chemical elicitors in the text



diffusible autoregulatory signaling molecule that controls development and streptomycin production in *S. griseus* at very low concentrations [55, 110]. The key enzyme that mediates the biosynthesis of A-factor is the product of the *afsA* gene product [65]. When A-factor reaches a critical level it binds to the TetR-family regulator ArpA, which consequently dissociates from the *adpA* promoter, thereby alleviating its repression. In turn, AdpA then globally *trans*-activates developmental and antibiotic genes [110]. Antimicrobial regulatory systems that involve γ-butyrolactones have been discovered in many *Streptomyces* species, controlling among others the production of Lankacidin and Lankamycin in *Streptomyces rochei* [165], virginiamycin production in *Streptomyces virginiae* [66], showdomycin and minimycin in *Streptomyces lavendulae* [69], auricin production by *Streptomyces aureofaciens* [104], and the biosynthesis of the type I modular polyketide Cpk in *S. coelicolor* A3(2) [34]. The activation of many other antibiotics is likely also mediated through γ-butyrolactones. A second *afsA*-like gene, *mmfL*, occurs in

S. coelicolor, which is involved in the biosynthesis of methylenomycin furans (MMFs; Fig. 3), controlling a signaling pathway involved in regulation of methylenomycin biosynthesis. Like GBLs, MMFs are synthesized via a butenolide intermediate [31].

Conceivably, γ-butyrolactones may be applied for drug discovery, as shown by surprising antibiotic stimulation in *Streptomyces natalensis* by the addition of A-factor from *S. griseus*. *S. natalensis* produces the autoinducer molecule PI factor (2,3-diamino-2,3-bis (hydroxymethyl)-1,4-butanediol; Fig. 3), which is required for the activation of pimaricin production in this organism [123]. Pimaricin is a 26-membered macrolide tetraene with antifungal activity [7]. Mutants that fail to produce PI factor can not only be restored to produce pimaricin by the addition of PI factor itself, but also by A-factor. While PI factor has a stronger stimulatory effect than A-factor, the cross-complementarity is remarkable. Another example of overlapping signaling routes was found for A-factor and the signaling protein Factor C [19] from *Streptomyces flavofungini* (originally regarded as a variant of *S. griseus*), although this time it is the lack of A-factor that is complemented. Factor C fully restored normal development and streptomycin production to an A-factor nonproducing strain (AFN) of *S. griseus*, even though *S. griseus* does not produce Factor C itself [20]. Factor C restored wild-type levels of A-factor production to the AFN mutant via a yet-unknown mechanism [18], and elucidation of this mechanism should shed new light on the way A-factor production is induced.

Little is yet known about possible application of these signaling molecules as global elicitors of antibiotic production, but considering their activity at very low concentrations this is an interesting approach to follow. Approaches to apply GBLs and MMLs for the activation of antibiotic production were recently proposed by Corre and coworkers [133], and the effect of the furan-like triclosan and the related ARC molecules (previous section) is further support for this conceptual idea.

Rare earth elements

Rare earth elements (REEs) have recently been implicated as activators of poorly expressed secondary metabolites [106, 108]. Scandium and/or lanthanum enhanced the production of actinomycin, actinorhodin, and streptomycin by two- to as much as 25-fold at 10–100 μM concentrations in *Streptomyces antibioticus*, *S. coelicolor*, and *S. griseus*, respectively. Moreover, scandium also activated actinorhodin in *S. lividans* [67, 143] and amylase and bacilysin production in *B. subtilis* [60]. REEs are widely distributed and microorganisms respond to their presence in their environment. Addition of REEs, and in particular scandium [106], during screening, may be a useful addition to the array of tools researchers have at their disposal to elicit the production of antibiotics.

HDAC inhibitors

Another recent addition to the arsenal of chemical elicitors are inhibitors of histone deacetylases or HDACs. Molecules that affect histone acetylation, and thereby change chromatin structure, were shown to activate biosynthetic clusters for natural products in fungi [21, 138]. HDACs antagonize the acetylation of histones in eukaryotes, leading to alterations in chromosome structure and thus affecting gene expression [137]. HDAC proteins are widespread and many are found in bacteria [76], with three HDAC-like genes identified in *S. coelicolor* [96]. Analysis of the effect of sodium-butyrate, a well-known HDAC inhibitor (Fig. 3), on antibiotic production by *S. coelicolor* showed a major effect on actinorhodin production [96]. Surprisingly, the response of *S. coelicolor* displayed a similar context-dependence as previously observed for *N*-acetylglucosamine [125], namely enhanced production of actinorhodin on minimal media ("famine") and repression under rich ("feast") growth conditions (Fig. 2b; [96]). Whether there is a correlation between the mechanisms by which *N*-acetylglucosamine and sodium-butyrate enhance antibiotic production awaits further investigation.

Genome mining

PKS and NRPS gene clusters can be readily identified using bioinformatics, and the natural products they specify can be predicted based on protein domain structures [9, 73]. In recent years, there has been a great expansion in bioinformatics programs enabling the identification of genes involved in secondary metabolite production. This includes ANTIbiotics and Secondary Metabolite Analysis SHell (antiSMASH) [93] and Secondary Metabolite Unknown Regions Finder (SMURF), which facilitate the automated detection of secondary metabolite biosynthesis gene clusters in genome sequence assemblies. Other software packages, such as CLUster SEquence ANalyzer (CLUSEAN), ClustScan, Structure Based Sequence Analysis of Polyketide Synthases (SBSPKS), NRPSPredictor, and Natural Product searcher (NP.searcher), enable the identification of secondary metabolite backbone biosynthesis genes [40]. Other packages include the NORINE database for nonribosomal peptides [23] and BAGEL, which is specific for the identification of biosynthetic clusters for bacteriocins and lantibiotics [35].

The rapid decline in cost made genome sequencing a feasible strategy for identification of gene clusters. Helped by the new software tools that have become available,

thousands of gene clusters have now been identified, and many more have undoubtedly been elucidated outside the public domain. However, how can we efficiently deal with this wealth of information? Expressing them one by one is like looking for the proverbial needle in a haystack, so how can we identify those clusters that may qualify as producing novel compounds? Following the initial excitement of the massive amount of new cluster data, we should not just focus on identifying homologs of known biosynthetic gene clusters. Instead, we should make use of computational techniques to intelligently sieve through the data for interesting new biochemistry. For example, all gene clusters may be subdivided into families using an evolutionary distance metric (Cimermancic, Medema, Fischbach et al., unpublished data). This allows one to focus on families without gene clusters encoding the biosynthesis of known compounds. Alternatively, one could focus on families that contain gene clusters with entirely novel combinations of homologs of well-known enzymes. Such gene clusters are likely associated with specific types of regulatory genes and/or regulatory elements, as well as genes for modifying enzymes and transporters. Genomic, transcriptomic, and/or proteomic data may be used as further support, e.g., to select those clusters that appear poorly expressed under routine growth conditions. Here we look at *cis*-acting elements and specific regulatory genes that may act as so-called "molecular beacons" [11], which may point scientists in the right direction in their search for novel antibiotic biosynthetic gene clusters.

Regulatory elements as beacons

In terms of scanning the genomes of yet un(der)explored streptomycetes, following the distribution of regulatory elements for global antibiotic regulators may be a useful strategy. As an example, scanning the *S. coelicolor* genome using the PREDetector algorithm [49] revealed some 200 sequences that conformed to the consensus binding site for DasR (*dre*, for DasR responsive element), namely the palindromic 16-bp consensus sequence A(G/C)TGGTCTA GACCA(G/C)T. The DasR regulatory network is well conserved in streptomycetes, with around 75 % of the DasR-binding (*dre*) sites predicted in *S. coelicolor* also found upstream of the orthologous genes in *S. avermitilis* (Rigali, Titgemeyer and van Wezel, unpublished data and [153]). Scanning genome sequences in the databases suggested that DasR may control the biosynthesis of novel antibiotics as well as important clinical drugs, including clavulanic acid, chloramphenicol, daptomycin, and teicoplanin.

A similar approach could be followed by analyzing the distribution of the regulatory element of AtrA, which among others controls actinorhodin production in *S. coelicolor* [148]. AtrA recognizes the consensus sequence cGGAA(T/C)(G/C)NNN(C/G)(A/G)TTCCg (are, for AtrA-responsive element) and likely qualifies as a global regulator (K.J. McDowall, pers. comm.). AtrA occurs in all streptomycetes, its DNA binding domain is extremely well conserved (>90 % aa identity), and an AtrA orthologue activates streptomycin production in *S. griseus* [50]. Following a similar approach to the DasR regulatory network should reveal how the AtrA network associates with (novel) secondary metabolite gene clusters. The same is true for other regulatory networks.

Regulatory genes as beacons

mbtH-family genes and NRPS

The gene *mbtH* was identified in *Mycobacterium tuberculosis* within the NRPS gene cluster for mycobactin, a peptide siderophore. The *mbtH*-like genes are widespread in *Streptomyces* genomes, and some of the gene products stimulate adenylation reactions by tightly binding to NRPS proteins containing adenylation (A) domains. Their expression may be important for the efficient production of native and recombinant secondary metabolites produced using NRPS enzymes [11]. As an example, *vbsG*, an *mbtH* homolog in *Rhizobium leguminosarum*, is required for the production of the cyclic, trihydroxamate siderophore vicibactin [24]. Thus, *mbtH* homologs can be useful for identification of specific types of NRPS gene clusters [11].

mmyB-family regulatory genes

The *mmyB*-like genes are also candidates as beacons for antibiotic biosynthetic gene clusters. *mmyB* itself is a transcriptional regulatory gene involved in the biosynthesis of methylenomycin in *S. coelicolor* [105]. The crystal structure of the MmyB-family regulator MltR from *Chloroflexus aurantiacus* was resolved; the proteins consist of an Xre-type N-terminal DNA-binding domain and a C-terminal ligand-binding module that is related to the Per-Arnt-Sim (PAS) domain, and these regulators most likely bind complex fatty acid molecules as ligands that activate their DNA binding activity [163]. Many *mmyB*-family regulatory genes lie divergently transcribed from, and share the promoter region with, genes related to antibiotic production. There are eight in total in *S. coelicolor*, including *mmyB* itself, which controls Mmy biosynthesis and SCO6925, which lies next to a lantibiotic biosynthetic cluster. The other genes are adjacent to a gene for an NAD(P)H-dependent short- or medium-chain dehydrogenases/reductase (SDR or MDR), a diverse family with alcohol dehydrogenase as the best known example [64]. This suggestive linkage is conserved in actinomycetes,

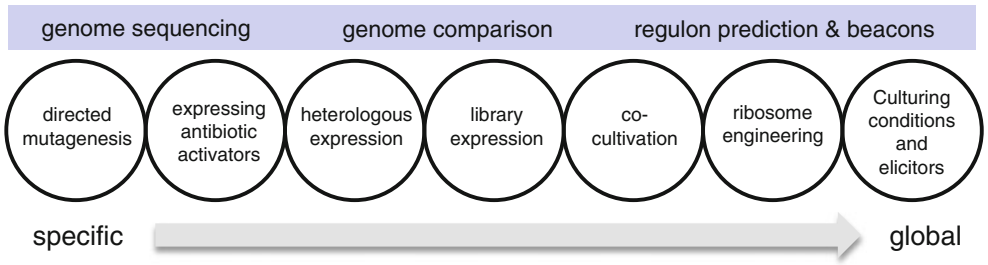


Fig. 4 Approaches to activate antibiotic production. Summary of methods applied to increase or induce the production of secondary metabolites. From *left* to *right*, technologies are presented with increasing applicability at higher throughput, from strain-specific (directed mutants, expression of activators) to HT screening using elicitors. Genome mining approaches range from single genome sequencing and annotation (*left*) to global genome comparison (*right*). For details on the technologies, see the text

suggesting that the genetic association with antibiotic-related genes appears to be widespread.

The *mmyB*-family gene SCO4944 is conserved in streptomycetes and other actinomycetes, and may therefore be seen as the main member of the family in actinomycetes. Phylogenetic evidence suggests that SCO4944 may control SCO4945, a gene for a mycothiol-dependent formaldehyde dehydrogenase. Surprisingly, the orthologs in *S. griseus*, SGR_6891 and SGR_6892, respectively, are separated by one gene from *afsA* (SGR_6889) for A-factor synthase, and their transcription is induced by A-factor immediately after its addition to liquid-grown cultures [48], suggesting that both genes are part of A-factor regulatory cascade. Suggestively, MmyB binds furans as ligands, which are structurally similar to A-factor.

Thus, *mmyB* homologs may form a very useful tool as genetic beacon for the identification of antibiotic-related genes in actinomycete genomes, and that searching for large gene clusters with suggestive genes such as modifying enzymes and transporters could be a fruitful strategy for finding new candidate antibiotic biosynthetic clusters.

Final considerations

With next-generation sequencing technologies and the increasing understanding of antibiotic regulation, new strategies for "awakening" poorly expressed antibiotics are becoming available. A combination of different regulatory approaches should be considered for activating antibiotic production, such as the application of elicitor molecules or transcription stimulation, to enhance the expression of novel biosynthetic gene clusters. Important approaches to explore also lie in the direction of culture conditions. The different methods range from strain-specific to globally applicable (Fig. 4). One obvious problem with routine screening methods is the absence of the natural competitors and symbionts that are found in the natural habitat. Co-cultivation methods or chemical mimicking of inter-species communication are promising new approaches in the search for novel antibiotics. One aspect that has obtained surprisingly little attention is that while most scientists search for novel antibiotics, the problem lies not so much in the lack of antibiotics, but in the widespread resistance that limits their application. The success story of clavulanic acid as inhibitor of β-lactamases [114, 122] perfectly illustrates the potential of such approaches. Therefore, targeting antibiotic resistance should offer a very attractive alternative to antibiotic discovery. We anticipate that similar considerations as those described above for antibiotics will also be applicable to the activation of natural products involved in counteracting antibiotic resistance mechanisms.

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