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Research review paper

Elicitation of secondary metabolism in actinomycetes

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ABSTRACT

Genomic sequence data have revealed the presence of a large fraction of putatively silent biosynthetic gene clusters in the genomes of actinomycetes that encode for secondary metabolites, which are not detected under standard fermentation conditions. This review focuses on the effects of biological (co-cultivation), chemical, as well as molecular elicitation on secondary metabolism in actinomycetes. Our review covers the literature until June 2014 and exemplifies the diversity of natural products that have been recovered by such approaches from the phylum Actinobacteria.

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Contents

1. Introduction

Actinomycetes (Phylum: Actinobacteria) are well-known for their ability to produce a wealth of natural products with structural complexity and with diverse biological activities (Abdelmohsen et al., 2014a,b; Adegboye and Babalola, 2012; Hopwood, 2007; Li and Vederas, 2009; Nett et al., 2009). However, a plethora of secondary metabolites encoded in the actinomycete genomes remain undiscovered presumably because these genes are not transcribed under conventional laboratory conditions (Bentley et al., 2002; Hertweck, 2009; Ochi and Hosaka, 2013; Pettit, 2011; Seyedsayamdost, 2014; Van Lanen and Shen, 2006). Mathematical models predict thousands of unexplored antimicrobials from actinomycete genomes (Caboche, 2014; Cimermancic et al., 2014; Watve et al., 2001). Accordingly, significant research effort

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has been dedicated to de-silence the underlying gene clusters with the aim to identify new natural products and molecular scaffolds. Since the discovery of the β-lactam antibiotic penicillin by Alexander Fleming in 1928 (Fleming, 1929), co-cultivation or mixed fermentation has been recognized as a remarkably successful approach for the discovery of biologically active natural products. Several recent studies have revealed strategies to activate cryptic genes, which include chemical, biological, and molecular elicitation (Dashti et al., 2014; Gross, 2009; Hertweck, 2009; Luo et al., 2013; Schroeckh et al., 2009; Zhu et al., 2014). Traditional approaches such as co-cultivation and changes in fermentation conditions (media composition, pH, and temperature) are long known to induce significant changes in the microbial metabolome (Marmann et al., 2014). Manipulation of the fermentation conditions is known as "one strain many compounds" (OSMAC) approach and represents an effective avenue for activating silent or poorly expressed metabolic pathways (Abdelmohsen et al., 2014a,b; Bode et al., 2002; Paranagama et al., 2007; Wei et al., 2010). Co-cultivation has been used in the production of foods, food additives, enzymes, bulk and fine chemicals, bioremediation, and degradation of lignocelluloses. However, its application for the production of antimicrobial compounds is still in its infancy (Bader et al., 2010). Challenging the bacterial cells with external signals, so-called "elicitors", is a recognized strategy to generate novel, biologically active metabolites (Chiang et al., 2011; Müller and Wink, 2014). Sensing of the input signals by the producer strains results in a discrete response that might lead to an altered metabolite profile. The challenge with small molecule chemical elicitors can enable the identification of different responses in microorganisms such as the increased production of stress-response-related compounds (Romero et al., 2007), biofilm formation induction, and modulation of virulence expression (Bajaj et al., 2014; Even et al., 2009; Nouaille et al., 2009). Elicitation may, in certain rare cases, result in unaltered metabolite profiles or even repression of the sought-after metabolites (Marmann et al., 2014). There are several recent reviews on activating silent gene clusters in fungi (Brakhage, 2013; Brakhage and Schroeckh, 2011; Chiang et al., 2009), as well as in myxobacteria (Krug et al., 2008; Wenzel and Müller, 2009), but remarkably little is known about the activation of gene clusters in actinomycetes by elicitation approaches. Given that the large number of available actinobacterial genome sequences have shown the hidden natural product biosynthetic capacity of the phylum Actinobacteria (Bentley et al., 2002; Doroghazi et al., 2014; Jensen et al., 2014; Zazopoulos et al., 2003), our review will focus on elicitation of secondary metabolism in actinomycetes.

The chemical and molecular complexity of the elicitation process renders the precise mechanism of elicitation poorly understood. To date, the mechanism of the action of elicitors is best known in plants and fungal cells (Nützmann et al., 2011; Radman et al., 2003), but less well known than bacterial cells. The current knowledge on the postulated mechanistic underpinnings of elicitation in bacteria is summarized in Fig. 1. The elicitors are likely initially sensed by the bacterial cells as chemical signals (Kawai et al., 2007; Murphy et al., 2011). The binding of an elicitor to a receptor or its internalization may then lead to changes in the levels of intracellular molecules such as Ca^{2+} ions (Murphy et al., 2011), alarmones (Kawai et al., 2007), or reactive oxygen species (ROS) (Radman et al., 2006). These concentration changes serve as internal signals to direct the regulation of secondary metabolites via yet unknown mechanisms. The elicitor can either directly influence the transcription of the secondary metabolite gene cluster or induce a transcriptional activator of the target gene cluster (Kawai et al., 2007; Nair et al., 2009; Rigali et al., 2008; Tanaka et al., 2010).

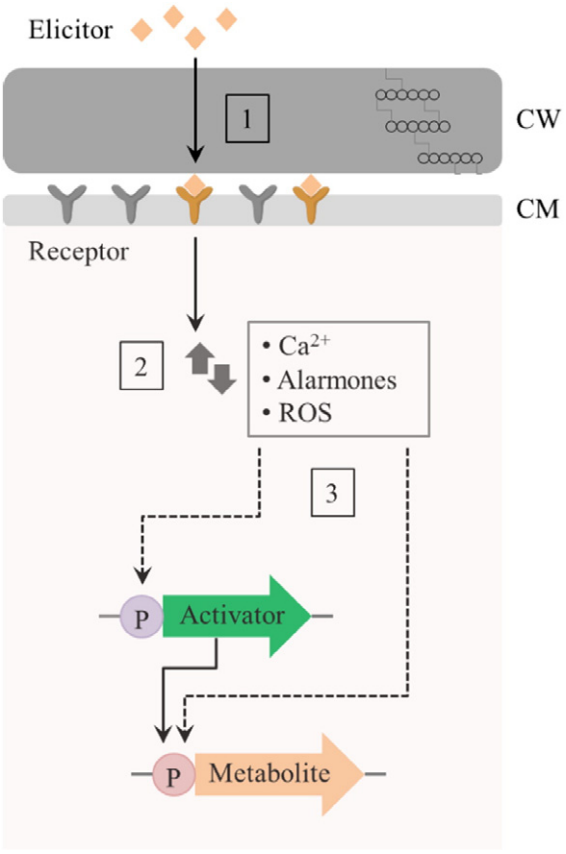


Fig. 1. Hypothetical mechanisms of elicitation of secondary metabolism in bacterial cells, the data have been derived from the already published literature: (1) Binding of elicitor to a specific receptor or its internalization; (2) changes in the levels of intracellular molecules as a response to the elicitors; (3) Activation of either the metabolite activator or the metabolite gene cluster itself (via unknown mechanisms). CW, cell wall; CM, cytoplasmic membrane and P, promoter.

2. Biological elicitation

2.1. Elicitation by microbial co-cultivation

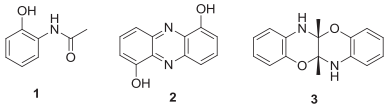
Actinomycetes exist as cohorts along with other microbes in various habitats. The encounter of microorganisms in their environment may turn on cryptic gene clusters, thereby triggering the production of new metabolites in the resident bacteria. This interaction could induce silent pathways that might result in the synthesis of novel secondary metabolites, and ultimately lead to chemical defense of the producing microorganism. Co-cultivation-based elicitation can therefore lead to the production of compounds that are not produced in monoculture, which may help to unmask cryptic and poorly expressed metabolites.

Even though the precise mechanisms of the interaction are rarely understood (Marmann et al., 2014), various scenarios are conceivable. For example, physical cell–cell interactions (Fig. 2A), production of small molecules (auto-regulator/quorum sensing molecules, siderophores, etc.), (Fig. 2B), production of enzymes that activate the metabolite precursor produced by the producer strain, yielding the active metabolite (Fig. 2C), or horizontal gene transfer (Fig. 2D) may affect metabolite activation or repression. The latter phenomenon happens on evolutionary time scales and any changes are permanently fixed on the recipient genome. Apart from these mechanisms, the inducer strain could also induce epigenetic (e.g. histone acetylation) modifications in the producer strain. In the above models, the interaction of the inducer strain with the producer strain ultimately influences the secondary metabolite production in the producer strain. However, the inducer and the producer strains are not easily identified unless specific experiments, for example, cross-feeding assays are performed. Co-cultivation strategy thus partly simulates a real time ecological scenario wherein microbes interact in a three dimensional space. Table 1 represents a comprehensive list of elicitation experiments with live cells (co-cultivation) and cell lysates involving actinomycetes along with the secondary

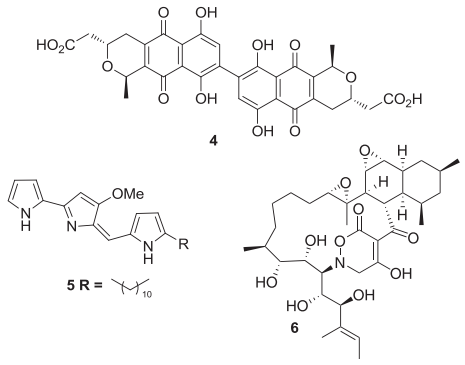
metabolites reported. Co-cultivation studies could be classified further based on the sets of strains being co-cultured as (a) co-cultivation of actinomycetes with other actinomycetes; (b) co-cultivation of actinomycetes with other bacteria, and (c) co-cultivation of actinomycetes with fungi. Each of the above three categories is further elaborated in the following section.

2.1.1. Co-cultivation of actinomycetes with actinomycetes

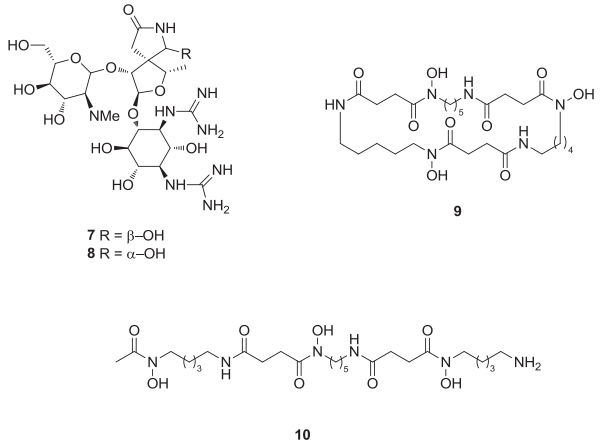
Co-cultivation of two marine sponge-associated actinomycetes, namely *Actinokineospora* sp. EG49 and *Nocardiopsis* sp. RV163, resulted in the production of three metabolites, which were not produced when the strains were grown independently. These metabolites were identified as *N*-(2-hydroxyphenyl)-acetamide (**1**), 1,6-dihydroxyphenazine (**2**), and 5a,6,11a,12-tetrahydro-5a,11a-dimethyl[1,4]benzoxazino[3,2-*b*][1,4]benzoxazine (**3**). Of the three compounds, phenazine **2** showed appreciable biological activity against *Bacillus* sp. p25 and *Trypanosoma brucei*, as well as against *Actinokineospora* sp. EG49 itself. These findings suggest that the newly synthesized molecules were produced by the strains in mixed culture (Dashti et al., 2014).



Alteration in the natural product biosynthesis occurred in different soil *Streptomyces* strains upon co-cultivation with the mycolic acid containing *Tsukamurella pulmonis* TP-B0596, *Rhodococcus erythropolis*, and *Corynebacterium glutamicum*. Production of red pigments corresponding to the benzoisochromanequinone polyketide actinorhodin (**4**) and the tripyrrole undecylprodigiosin (**5**) by *Streptomyces lividans* TK-23 was used as an indicator to target the inducer strain. Only *T. pulmonis* TP-B0596 out of 400 tested inducer strains was able to stimulate the production of the red pigments by *S. lividans* TK-23. A dialysis experiment confirmed that this effect was a consequence of physical cell–cell interactions. Members of the genus *Corynebacterium*, as well as strains closely related to *T. pulmonis* (*Tsukamurella pseudospumae*, *Tsukamurella spumae*, *Tsukamurella strandjordii*) showed a similar effect in stimulating the production of the red pigments by *S. lividans* TK-23. The role of mycolic acid was confirmed by the use of a mycolic acid biosynthesis deletion mutant (pks 13) of *C. glutamicum* that was unable to activate red pigment production in *S. lividans*. Furthermore, upon treatment of the co-culture with isoniazid, a mycolic acid synthase inhibitor, the red pigment production by *S. lividans* TK-23 was not activated, thus highlighting the efficacy of mycolic acid in activating the red pigment production, although mycolic acid per se had no effect. In addition, the combined culture of a *Steptomyces endus* S-522 with *T. pulmonis* resulted in the production of a novel antibiotic alchivemycin A (**6**) with activity against *Micrococcus luteus*. The compound was produced only in co-culture. Hence, co-cultivation with mycolic acid containing bacteria is one elegant way to activate silent gene clusters (Moody, 2014; Ochi and Hosaka, 2013; Onaka et al., 2011).



Co-culture of a multi-antibiotic resistant mutant strain of *Rhodococcus fascians* with *Streptomyces padanus* led to the emergence of a new strain *Rhodococcus* 307CO that was found to encompass a large segment of genomic DNA from *S. padanus* (Kurosawa et al., 2008). Fermentation of *Rhodococcus* 307CO in the production medium led to the synthesis of optically active, hydrophilic isomers of a new class of aminoglycosides, rhodostreptomycins A (**7**) and B (**8**). The compounds were not produced by the mutant strain of *R. fascians*, which implies the essential role of horizontal gene transfer in the synthesis of the rhodostreptomycins. Antimicrobial assays against a panel of microbes (*S. padanus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Helicobacter pylori*, and *Saccharomyces cerevisiae*) demonstrated that β-isomer **7** was more active than the α form **8** (Kurosawa et al., 2008). Even though the exact mechanism of horizontal gene transfer from *S. padanus* to *R. fascians* remains unknown, antibiotic synthesis followed by ways of horizontal gene transfer provides a novel avenue for elicitation of novel metabolites in actinomycetes.



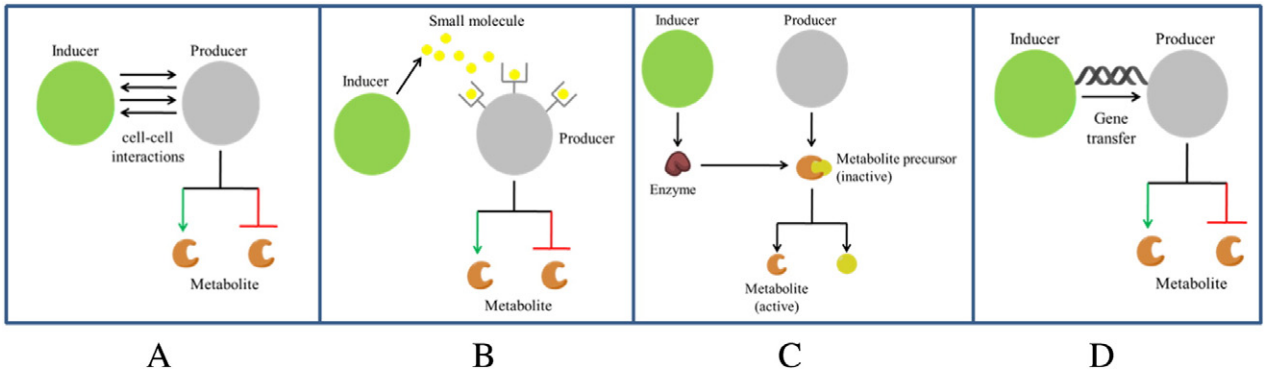


Fig. 2. Putative consequences of co-cultivation: (A) physical cell to cell interactions, (B) small molecule-mediated interactions, (C) catalytic activation of metabolite precursor and (D) horizontal gene transfer.

Table 1
Comprehensive list of secondary metabolites reported upon elicitation with live cells (co-cultivation) and cell lysates.

Interaction partners	Induced secondary metabolites	Activity	Reference
A — Elicitation with live cells			
Actinomycetes–Actinomycetes	N-(2-hydroxyphenyl)-acetamide (**1**)	–	Dashti et al. (2014)
Actinokineospora sp. EG49 (U) *Nocardiopsis* sp. RV163 (U)	1,6-dihydroxyphenazine (**2**) 5a,6,11a,12-tetrahydro-5a,11a dimethyl[1,4]benzoxazino[3,2b][1,4]benzoxazine (**3**)	A, AT –	
Steptomyces endus S-522 (P) *Tsukamurella pulmonis* TP-B0596 (I)	Alchivemycin A (**6**)	A	Moody (2014), Ochi and Hosaka (2013) and Onaka et al. (2011)
Rhodococcus 307CO (P) Co-culture of *Rhodococcus fascians*, and *Streptomyces padanus*	Rhodostreptomycin A (**7**) Rhodostreptomycin B (**8**)	A A	Kurosawa et al. (2008)
Streptomyces tanashiensis IAM0016 (P) *Streptomyces griseus* st-21-2 (I)	Unknown antibiotics against *B. subtilis*	A	Yamanaka et al. (2005)
Streptomyces coelicolor M145 (P) *Amycolatopsis* sp. AA4 (I)	γ-Actinorhodin (**11**), Prodiginine (**12**) Acyl-desferrioxamines (**14**–**17**)	A IS	Moody (2014) and Traxler et al. (2013)
Streptomyces sp. E14 (I)	Acyl-desferrioxamines (**14**–**17**)	IS	
Streptomyces sp. SPB74 (I) *Streptomyces virdiochromogenes* (I)	Actinorhodin (**11**), Prodiginine (**12**) Acyl-desferrioxamines (**14**–**17**)	IS IS	
Streptomyces albus (I)	Actinorhodin (**11**), Prodiginine (**12**), Desferrioxamine E (**9**) Actinorhodin (**11**), Prodiginine (**12**), Desferrioxamine E (**9**), Desferrioxamine B (**10**)	IS	
Actinomycetes–other bacteria *Streptomyces coelicolor* A3 (2) (P) *Bacillus subtilis* (I)	Undecylprodigiosin (**5**)	A	Luti and Mavituna (2011)*
Streptomyces coelicolor (P) *Myxococcus xanthus* (I)	Actinorhodin (**4**)	A	Perez et al. (2011)
Streptomyces griseorubiginosus 43708 (P) *Pseudomonas maltophilia* 1928 (I)	Biphenomycin A (**18**) Biphenomycin C (**19**)	A A	Ezaki et al. (1992) and Ezaki et al. (1993)
S. coelicolor M145 (P) *Corallococcus coralloides* B035 (I)	Undecylprodigiosin (**5**)	A	Schäberle et al. (2014)
Streptomyces tejamariensis SS-939 (ATCC 31603) (P) 12 unidentified bacteria (I)	Istamycin (**20**)	A	Slattery et al. (2001)
Actinomycetes–Fungi *Aspergillus fumigatus* (P) *Streptomyces peucetius* ATCC 29050 (I)	Sulfated formyl xanthocillin analogue Fumiformamide (**21**) N,N′-((1Z,3Z)-1,4-bis(4-methoxyphenyl)buta-1, 3-diene-2,3-diyl)diformamide (**22**) N-formyl derivatives (**23**, **24**) Xanthocillin analogue BU-4704 (**25**)	CT(−) CT CT(−), CT CT(−)	Zuck et al. (2011)
Emericella sp. (strain CNL-878) (P) *Salinispora arenicola* (strain CNH-665) (I)	Emericellamides A (**26**) Emericellamides B (**27**)	A, CT A, CT	Oh et al. (2007)
Aspergillus nidulans (P *Streptomyces rapamycinicus* (I)	Orsellinic acid (**28**) Lechanoric acid (**29**) Cathepsin-K inhibitor F-9775A (**30**) Cathepsin-K inhibitor F-9775B (**31**)	NT NT NT NT	Chiang et al. (2011) and Schroeckh et al. (2009)
Aspergillus fumigatus (P) *Streptomyces rapamycinicus* (I)	Fumicycline A (**32**) Fumicycline B (**33**)	A A	König et al. (2013) and Marmann et al. (2014)
Aspergillus fumigatus MBC-F1-10 (P) *Steptomyces bullii* (I)	Ergosterol (**34**) Brevianamide F (**35**) Spirotryprostatin A (**36**) 6-methoxyspirotryprostatin B (**37**) Fumitremorgin C (**38**) 12,13-Dihydroxyfumitremorgin C (**39**) Fumitremorgin B (**40**) Verruculogen (**41**) 11-O-methylpseurotin A (**42**) 11-O-methylpseurotin A2 (**43**)	NT CT AT, L, CT L, CT AT, L, CT AT, L, CT AT, L, CT AT, L, CT CT L, CT	Marmann et al. (2014) and Rateb et al. (2013)
Fusarium tricinctum (P) *Streptomyces lividans* (I)	Enniatin B (**47**) Enniatin B1 (**48**) Enniatin A1(**49**) Fusaristatin A (**50**) 4 unknown metabolites	– A A – NT	Ola et al. (2013)
B—Elicitation with cell lysates			

(continued on next page)

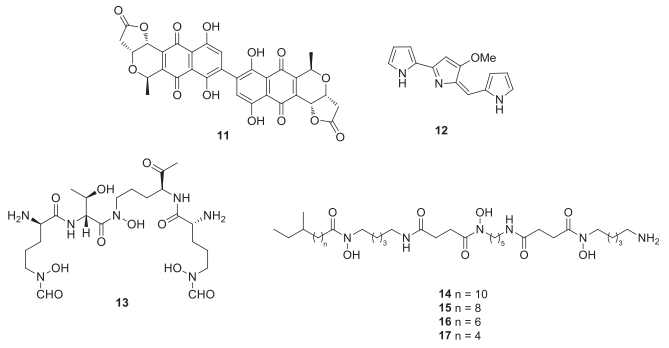
Table 1 (*continued*)

Interaction partners	Induced secondary metabolites	Activity	Reference
Streptomyces coelicolor A3(2) (P) Heat-killed/dead cells of *Bacillus subtilis* (I)	Undecylprodigiosin (**5**)	A	Luti and Mavituna (2011) and Anon. (2011)*
Streptomyces coelicolor A3(2) (P) Heat-killed/dead cells of *Staphylococcus aureus* (I)	Undecylprodigiosin (**5**)	A	Luti and Mavituna (2011)*

P = producer strain; I = inducer strain; A = antibiotic; AT = antitrypanosomal; L = leishmanicidal; CT = cytotoxic; CT (−) = no cytotoxicity; NT = not tested; – = inactive; U = producer/inducer strain unknown; IS = iron siderophore induction. *Elicitation efficiency using live/dead cells as elicitors is approximately similar.

Cross-feeding assays showed that a diffusible factor, later on identified as the siderophore desferrioxamine E (**9**), from *Streptomyces griseus* st-21-2 stimulated growth, altered morphological development, and induced antibiotic production in *Streptomyces tanashiensis* IAM0016 (Yamanaka et al., 2005). Treatment of *S. griseus*, *Streptomyces coelicolor* A3 (2) and five soil actinomycetes with compound **9** showed a similar pattern in that aerial growth, morphological changes, pigment, and antibiotic production were stimulated, while treatment with the commercially available desferrioxamine E analogue desferrioxamine B (**10**) showed a much weaker stimulation. Disruption of the biosynthesis genes of **9** in the *des* operon of *S. coelicolor* A3(2) and subsequent restoration of the *des* mutant by exogenous supply of **9** suggested the effect of siderophores in activation of *S. coelicolor* A3 (2) growth, development and secondary metabolism. None of the other known siderophores displayed a stimulatory effect on *S. tanashiensis* IAM0016, indicating siderophore specificity for this strain. Therefore, siderophores produced by microbial neighbors can induce growth, development and secondary metabolism in siderophore defective strains.

The chemical response of *S. coelicolor* M145 to the interactions in a co-culture with 5 initiator strains (*Amycolatopsis* sp. AA4 (A), *Streptomyces* sp. E14 (E), *Streptomyces* sp. SPB74 (S), *Streptomyces virdiochromogenes* (V) and *Streptomyces albus* (J)) was deciphered employing Nano-DESI MS (nanospray desorption electrospray ionization mass spectrometry) and MALDI-TOF (matrix-assisted laser desorption ionization-time-of-flight) imaging mass spectrometry. *S. coelicolor* M145 and the initiator strains were spotted 5 mm apart on an agar plate. Spectral networking revealed an array of 629 compounds as a consequence of these interactions. Antibiotics such as γ-actinorhodin (**11**) and prodiginine (**12**) were stimulated in *S. coelicolor* M145 upon interaction with the A, S, V, J strains but not strain E. Genome sequencing of these initiator strains revealed the absence of antibiotic **11** and **12** biosynthetic genes indicating that these are produced by *S. coelicolor* M145 only in the co-culture. The NRPS siderophore peptide coelichelin (**13**) was found only occasionally except in the interaction between *S. coelicolor* M145 and the other five strains with V strain. Furthermore, the gene clusters or genes for coelichelin biosynthesis were identified in V, which raises the possibility of this molecule being produced by either of the strains upon interaction. Production of the siderophore desferrioxamines of varying acyl chain lengths (**14**–**17**) were observed in *S. coelicolor* (in the Nano-DESI spectral networks) upon interaction with siderophore-producing neighboring strains that facilitate a competition of iron between the species. Acyl-desferrioxamines (Acyl-DFO) were observed in the interactions with strains A, E, and S, and not with V and J. In the interactions with the strains V and J, the cyclic siderophore desferrioxamine E (**9**) was observed, while the linear analogue desferrioxamine B (**10**) was detected in the interaction with J. Furthermore, A and E were found to have no operons for desferrioxamine synthesis in their genomes like the other initiator strains, indicating that the production of desferrioxamines in these interactions is solely due to *S. coelicolor*, which was not the case with the other initiator strains (Moody, 2014; Traxler et al., 2013). Hence, emerging new analytical techniques such as Nano-DESI MS and MALDI-TOF to study the chemical communication in an actinomycete co-culture constitute a modern strategy in understanding the complex patterns in metabolite shifts and focus on novel metabolites in complex interaction schemes.

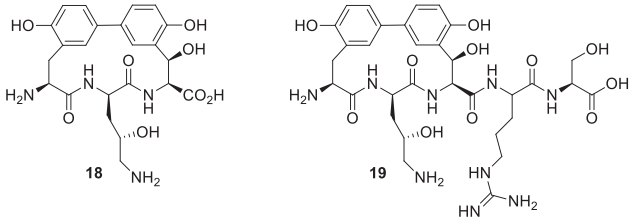


2.1.2. Co-cultivation of actinomycetes with other bacteria

Predator microbes that kill and feed on other live bacterial cells by absorbing their nutrients can also activate the expression of antibiotics in producer organisms. *Myxococcus xanthus*, a predator of *S. coelicolor*, was shown to induce the blue polyketide antibiotic actinorhodin (**4**) in the producer organism, as well as trigger aerial mycelium production on solid media (Perez et al., 2011). Furthermore, actinorhodin-producing cells were not surrounded by *M. xanthus* indicating that *S. coelicolor* could use compound **4** as a repellant signal against *M. xanthus*. Production of compound **4** was also upregulated in the presence of other strains such as *Bacillus megaterium*, *B. subtilis*, *Bacillus thuringensis*, and *Serratia* sp., however, the strongest upregulation was observed with *M. xanthus*. Liquid co-cultures of *S. coelicolor* with *M. xanthus* DK1622 and *S. coelicolor* with *M. xanthus* DZF1 resulted in a 20-fold elevation in the production of **4** when compared to *S. coelicolor* monoculture. Thus, the use of predator strains is another way to unravel the expression of metabolites that remain silent or poorly expressed in axenic cultures.

Co-culture of the producer strain *Streptomyces griseorubiginosus* 43708 and *Pseudomonas maltophilia* 1928 resulted in a 60-fold increase of the cyclic peptide antibiotic biphenomycin A (**18**) when compared to the pure culture that produced low levels of **18**. Treatment of *S. griseorubiginosus* 43708 with the cell free extract of *P. maltophilia* 1928 also led to the production of peptide **18**. This implied that the increase in production of **18** was not a consequence of mere cell–cell interaction but rather due to the presence of specific enzymes in the mycelia of strain 1928. These enzymes converted the precursor of biphenomycin A to its active form **18** in the co-culture and these enzymes were thought to be absent in the producer strain owing to production of low levels of the compound (Ezaki et al., 1992). Later on, the peptide precursor of **18**, biphenomycin C (**19**) (composed of **18** and arginylserine residue), was isolated and structurally identified from the mixed culture. Experiments

confirmed that an enzyme from *P. maltophilia* 1928 in the mixed culture converts the precursor **19** to **18** and arginylserine. Precursor **19** also possessed anti-bacterial activity against Gram-positive pathogens but the activity was lower when compared to that of **18** (Ezaki et al., 1993). Thus, the presence of certain enzymes in the inducer strains could convert precursors produced by producer strains to the active metabolite, leading to accumulation of novel metabolites in the co-culture and not in the mono-culture that are lacking these enzymes.

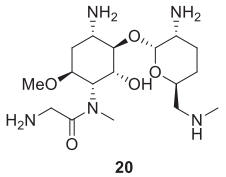


18 **19**

Co-cultivation of the soil bacterium *S. coelicolor* M145 with the corallopyronin A-producing myxobacterial strain *Corallococcus coralloides* B035 resulted in an increased production of the bioactive metabolite undecylprodigiosin (**5**) by *S. coelicolor* M145 when compared to the axenic culture (Schäberle et al., 2014). Treatment of *S. coelicolor* M145 with live or dead cells, or watery extract of *C. coralloides* B035 caused a 1.5-fold increase in the extracellular concentration and a 60-fold increase in the intracellular concentration of **5**. Elicitation of undecylprodigiosin in this co-cultivation approach was due to a hydrophilic and thermo-stable molecule from *C. coralloides* B035 that served as the signal for undecylprodigiosin production. This highlights the use of myxobacterial competitors as biotic elicitors in enhancing the production of bioactive metabolites. Changes that occurred during the co-cultivation can influence the expression of metabolites.

Co-cultivation of the soil microorganisms *S. coelicolor* M145 and *Pseudomonas fluorescens* BBc6R8 revealed that the acidification of the R2 medium by *P. fluorescens* BBc6R8 inhibited the production of the diffusible, blue pigmented antibiotic γ-actinorhodin (**11**) without altering the intracellular red pigmented actinorhodin (**4**) by *S. coelicolor* M145. This inhibition in the production of the lactone form of actinorhodin was attributed to a reduction in the pH of the medium due to synthesis of gluconic acid from glucose. A similar effect was observed with *S. coelicolor* M145 in co-culture with gluconic acid-producing bacteria like fluorescent *Pseudomonas* strains, the opportunistic pathogen *Pseudomonas aeruginosa* PAO1 and the β-proteobacteria *Collimonas* PMB3 (Galet et al., 2014). Thus, pH changes during mixed fermentation can affect the metabolite expression, and in consequence, modulate the expression of unknown metabolites.

The influence of competition between 53 marine bacteria with the istamycin (**20**) producing marine bacterium *Streptomyces tejamariensis* SS-939 was investigated to study the role of the antibiotic istamycin in competitive interactions between microbes under natural conditions. Twelve of the 53 strains induced the production of istamycin (**20**) in co-culture with *S. tejamariensis* SS-939. The co-cultures doubled the production of **20** when compared to the axenic mono-culture of *S. tejamariensis* (Slattery et al., 2001).



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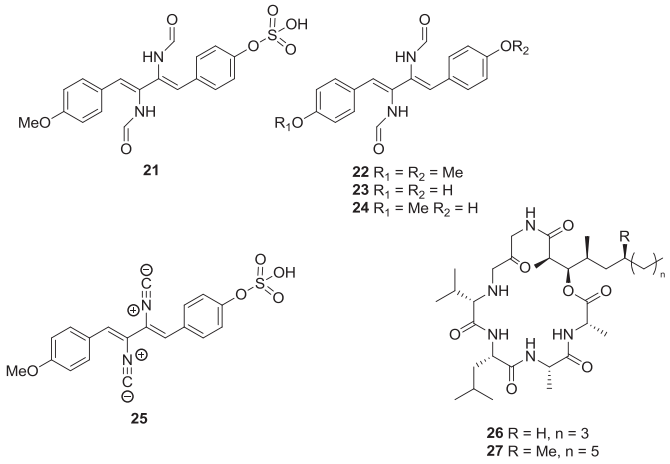
2.1.3. Co-cultivation of actinomycetes with fungi

Mixed fermentation of *Streptomyces peucetius* ATCC 29050 and *Aspergillus fumigatus* yielded two new metabolites, namely the sulfated formyl xanthocillin analogue fumiformamide (**21**) and *N,N′*-((1*Z*,3*Z*)-1,4-bis(4-methoxyphenyl)buta-1,3-diene-2,3-diyl)diformamide (**22**) along with two previously known *N*-formyl derivatives **23**, and **24**, as well as the new xanthocillin analogue BU-4704 (**25**) (Isaka et al., 2007). Compound **22** exhibited toxic effects on a broad spectrum of cell lines and compound **24** displayed moderate cytotoxicity, while no cytotoxic effects were observed for the other compounds (Zuck et al., 2011).

Anti-MRSA (Multi-drug Resistant *S. aureus*) cyclic depsipeptides, emericellamides A (**26**) and B (**27**) were obtained from the marine derived fungus *Emericella* sp. strain CNL-878 in co-culture with the marine actinomycete *Salinispora arenicola* strain CNH-665. Axenic culture of the *Emericella* sp. produced undetectable amounts of metabolites **26** and **27**. The co-culture of *Emericella* sp. with *S. arenicola* elevated the production of metabolites **26** and **27** by 100-fold and made the structural elucidations of these compounds feasible. Emericellamides A and B had moderate activities against MRSA, as well as weak cytotoxicity against HCT-116 human colon carcinoma cell lines (Oh et al., 2007).



21

22 $R_1 = R_2 = Me$
23 $R_1 = R_2 = H$
24 $R_1 = Me$ $R_2 = H$

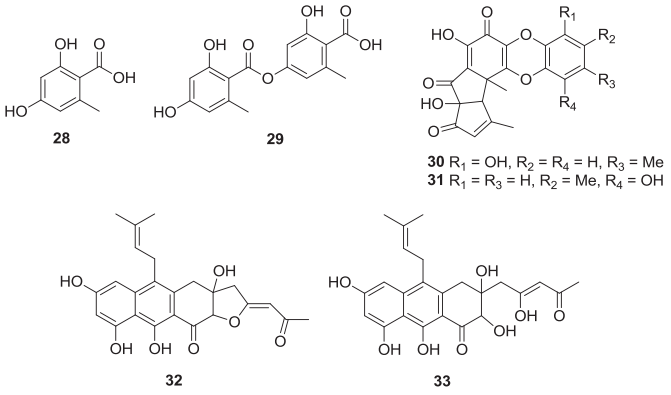
25

26 R = H, n = 3
27 R = Me, n = 5

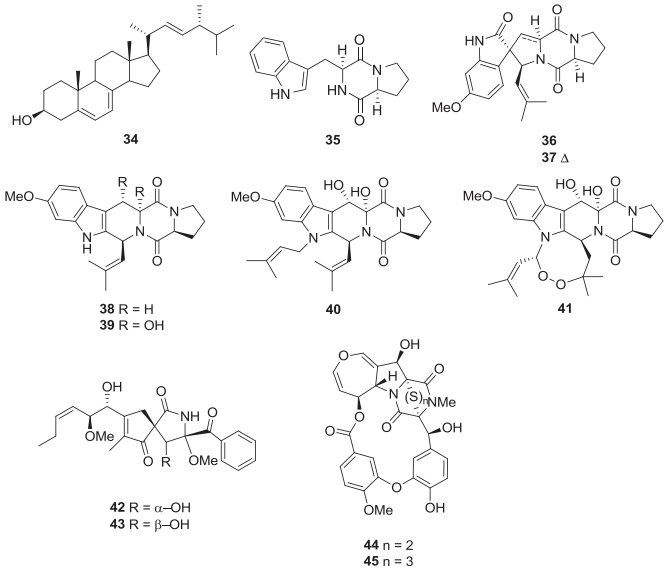
Specific polyketides were produced in *Aspergillus nidulans* upon co-culture with soil dwelling actinomycetes. *A. nidulans* secondary metabolism array (ASMA), microarray, quantitative RT-PCR, northern blot and full genome array results indicated that the bacteria, upon physical contact, activate certain fungal genes. Only *Streptomyces hygroscopicus* (ATCC 29253) among 58 bacterial soil isolates elicited the expression of two secondary metabolite gene clusters in *A. nidulans*. Transcriptome analysis revealed differential expression of about 395 fungal genes in the co-culture, of which 248 and 147 genes were significantly up- and down-regulated, respectively. HPLC analysis of the co-culture inferred the production of polyketides orsellinic acid (**28**), lechanoric acid (**29**), and cathepsin-K inhibitors F-9775A (**30**) and F-9775B (**31**). Furthermore, dialysis and the electron microscopy experiments revealed that the physical contact of the *S. hygroscopicus* with *A. nidulans* elicited metabolite production in the fungal cells and not the low molecular weight signaling molecules from the bacterial cells (Chiang et al., 2011; Schroeckh et al., 2009). This indicates that physical interaction of microorganisms is required to elicit expression of silent gene clusters in specific interaction sets. Differential gene expression in *A. nidulans* upon physical interaction with the *S. hygroscopicus* (*Streptomyces rapamycinicus*) is a consequence of fungal histone modification via the Histone Acetyl Transferase (HAT) complex Saga/Ada containing the proteins GcnE and AdaB that induces the orsellinic acid gene cluster (König et al., 2013; Nützmann et al., 2011).

A similar co-culture study of *S. rapamycinicus* and *A. fumigatus* led to the activation of novel C-prenylated polyketides fumicyclines A (**32**) and B (**33**) in *A. fumigatus*. Full genome microarray analysis revealed the upregulation of a large number of fungal genes. Agar diffusion assay against *S. rapamycinicus* showed the ability of **32** and **33** to act

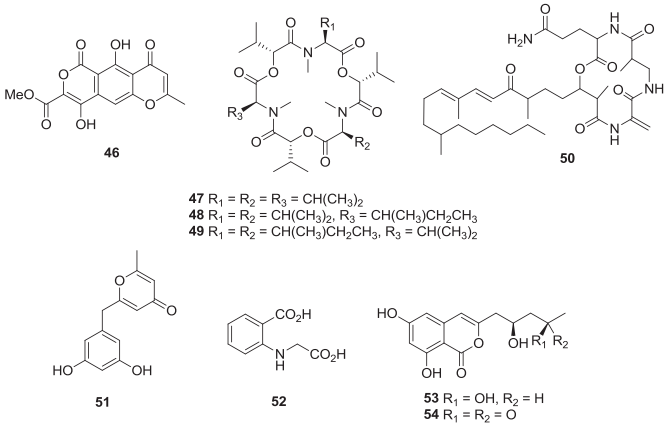
as fungal defense molecules, since both showed significant anti-bacterial activity against *S. rapamycinicus*. Dialysis experiments and experiments involving addition of bacterial cell free supernatants proved that direct physical contact between *S. rapamycinicus* and *A. fumigatus* was essential for metabolite induction (König et al., 2013). Hence, physical interaction between microbial sets regulates gene expression, facilitating the production of bioactive metabolites as a defense strategy aimed at survival in a competitive environment.



Quorum sensing molecules could also induce the secondary metabolite production in actinomycetes. Metabolites possessing antiprotozoal activities were induced in a co-culture of *A. fumigatus* MBC-F1-10 with the soil isolate *Streptomyces bullii* strain C2. Compounds of fungal origin including triterpene ergosterol (**34**), diketopiperazine alkaloids brevianamide F (**35**), spirotryprostatin A (**36**), 6-methoxyspirotryprostatin B (**37**), fumitremorgin C (**38**) and its 12,13-dihydroxy derivative (**39**), fumitremorgin B (**40**), verruculogen (**41**), 11-O-methylpseurotin A (**42**) and the epimer 11-O-Methylpseurotin A2 (**43**) were reported in the co-culture and were not observed in the axenic cultures. Neither the medium devoid of bacteria nor the methanolic extract of bacterial cells or the heat inactivated bacterial cell fraction elicited the metabolite response in the co-culture. Treatment of *A. fumigatus* MBC-F1-10 with the bacterial quorum sensing molecule *N*-butyryl-DL-homoserine lactone resulted in the production of di- and tri-sulfides emestrins A and B (**44** and **45**) in the fungal culture. Fumitremorgin B (**40**) and verruculogen (**41**) exhibited potency against *T. brucei* and *Leishmania donovani* but also pronounced cytotoxicity against human lung fibroblasts MRC5 cells (Rateb et al., 2013). Thus, interactions in a co-culture, as well as the differential response to the bacterial quorum sensing molecules could trigger new metabolite production.



Co-culture of the plant endophytic fungus *Fusarium tricinctum* with *B. subtilis* 168 *trpC2* on the solid rice medium led to a 78-fold increase in lateropyrone (**46**), enniatin-type cyclic depsipeptides enniatins B (**47**), B1 (**48**), and A1(**49**), as well as the lipopeptide fusaristatin A (**50**), over the axenic fungal culture. The same co-culture experiment yielded three new natural products, namely macrocarpon C (**51**), 2-(carboxymethylamino) benzoic acid (**52**), and (−)-citreoisocoumarinol (**53**) along with (−)-citreoisocoumarin (**54**). The four compounds were detected only in the co-culture and not in either mono-culture. Co-culture of *F. tricinctum* with *S. lividans* showed a similar increase in the accumulation of compounds **47**–**50**. Additionally, four new unidentified metabolites were reported that were different from the four metabolites produced in the co-culture of *F. tricinctum* with *B. subtilis* 168 *trpC2*. These results indicate the specific response of the fungus to different bacterial cells. Only the cyclic depsipeptides enniatin A1(**49**) and enniatin B1 (**48**) had anti-bacterial activities against *B. subtilis* 168 *trpC2* with an MIC of 8 and 16 μg/ml, respectively, as well as against pathogens *S. aureus*, *Streptococcus pneumoniae*, and *Enterococcus faecalis*, while enniatin B (**47**) was inactive. Lateropyrone (**46**) also showed significant activity against *B. subtilis* 168 *trpC2*, *S. aureus*, *S. pneumoniae*, and *E. faecalis* (Ola et al., 2013).



2.2. Elicitation with microbial lysates

The yield of undecylprodigiosin (**5**), an antibiotic with immunosuppressive properties and apoptotic effects against breast cancer cells (Anon., 2011; Ho et al., 2007; Luti and Mavituna, 2011; Williamson et al., 2006) by *S. coelicolor* A3 (2) was increased upon treatment with live and heat killed cells of *B. subtilis*, while pure cultures of *S. coelicolor* A3 (2) produced only extremely low concentrations of **5**. Undecylprodigiosin was produced on solid medium at the interface of the *B. subtilis* (inducer strain) and the *S. coelicolor* A3 (2) (producer strain), but not by the producer strain alone. This indicated that *B. subtilis* induced the production of **5** in *S. coelicolor* A3 (2). Moreover, the cell wall components (N-acetyl glucosamine and the peptidoglycan) did not result in induction of **5**, while the entire cell lysate induced its production (Luti and Mavituna, 2011). Because a specific elicitor molecule could not be identified, the exact mechanism remains unknown. In another study, production of compound **5** was elicited following addition of the heat killed cells of *B. subtilis* and *S. aureus* at the time of inoculation along with the *S. coelicolor* A3 (2) (producer strain). This resulted in a 3- and 5-fold increase in the production of **5** in the *B. subtilis* and *S. aureus* treated cultures, respectively. The higher yield with heat-killed *S. aureus* over *B. subtilis* may relate to the fact that the human-skin associated *S. aureus* is more foreign to *S. coelicolor* than *B. subtilis*, the latter of, which are natural soil dwellers (Luti and Mavituna, 2011). Hence, simulating the presence of a competing environment

with a biotic elicitor is a modern approach and can be used as driving force for cryptic bioactive metabolite gene expression in actinomycetes.

2.3. Elicitation with microbial cell components

Cell wall constituents, microbial hormones or signaling molecules, carbohydrates or biopolymers derived from cells could be employed as elicitors in provoking secondary metabolism. Table 2 shows a comprehensive list of cell-derived elicitors that stimulate secondary metabolism in actinomycetes.

Nutritional status of the microbial milieu might also influence the production of antibiotics. The cell wall component and the chitin monomer N-acetylglucosamine (GlcNAc) elicited the production of actinorhodin (**4**) and undecylprodigiosin (**5**) by *S. coelicolor* under poor nutrient conditions, whereas GlcNAc impeded the antibiotic production under rich nutrient conditions. This nutrient-based elicitation of GlcNAc was found to be associated with the DNA binding protein DasR. DasR responsive elements (*dre*) (the DNA binding sites of DasR) were found upstream to the activators of **4** and **5** (*redZ*). Electrophoretic mobility assays and RT-PCR analysis demonstrated that the binding of DasR to the *dre* regions upstream to *actII-ORF4* and *redZ*, down-regulated the antibiotic production. Challenging *S. coelicolor* with GlcNAc under famine conditions caused a derepression of antibiotic production by DasR mutant due to the allosteric binding of GlcN-6P (glucosamine-6-phosphate) to DasR. Enhanced transcript levels of '*kasO*', an activator of a cryptic gene cluster, was observed in the case of the DasR mutant *S. coelicolor* BAP29, highlighting the role of DasR in silencing gene clusters by repression of the corresponding activator of the cluster. Accordingly, interference with DasR activity could induce antibiotic production. GlcNAc induced antibiotic production in *Streptomyces clavuligerus*, *Streptomyces collinus*, *S. griseus*, *S. hygroscopicus* and *Streptomyces venezuelae*, when grown on poor nutrient medium, indicating their widespread antibiotic elicitation effect within the *Streptomyces* genus (Ochi and Hosaka, 2013; Rigali et al., 2008; Zhu et al., 2014). Thus, treatment of actinomycetes with GlcNAc under specific nutrient conditions is one novel way to awaken cryptic secondary metabolism gene operons.

Biosynthesis of the anti-fungal polyethylene antibiotic natamycin or pimaricin (**55**) by *Streptomyces natalensis* HW-2 was enhanced upon elicitation with a structurally unidentified fungal elicitor from *Penicillium chrysogenum* AS 3.5163. Both, the heat killed biomass and the fermentation broth of *P. chrysogenum* AS 3.5163 enhanced the production of **55**. This fungal elicitor from *P. chrysogenum* AS 3.5163 enhanced the yield of natamycin as much as 200% when compared to the untreated control. Primary quantitative analysis revealed that the elicitor from the fermentation broth of *P. chrysogenum* AS 3.5163 was found to be a low molecular weight molecule with polarity comparable to that of butanol (Wang et al., 2013).

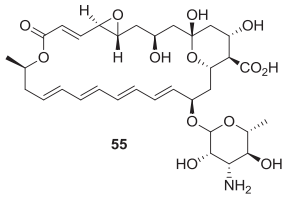
Cross-feeding experiments and pairing analysis of strains of *Streptomyces* sp. was done in three sets with *Streptomyces* strains from a culture collection and freshly isolated *Streptomyces* strains from two different soil environments. Higher frequency of cross-activation of antibiotic production and sporulation reported in pairwise strains of the fresh isolate reflected the higher incidence of such interactions in their natural habitats. The higher frequency of antibiotic production and sporulation in the pairs from culture collection was attributed to the genetic diversity of the strains in the collection. Externally added *Streptomyces* auto-regulators like A-factor or the virginae butanolide-C or γ-nonalactone did not activate the antibiotic production. This demonstrated that different factors were produced upon interactions of strains. The stimulation of antibiotics in the receiver strain by the ethyl acetate extracts of stimulator strains explains that the unknown diffusible factors might lead to antibiotic synthesis (Ueda et al., 2000).

Microbial hormones such as γ-butyrolactones in *Streptomyces* sp. regulate secondary metabolism and strain morphology (Waters and Bassler, 2005). One such example is the pimaricin (natamycin) (**55**) production by *S. natalensis* that was found to be elicited by the hydrophilic molecule PI factor [2,3-diamino-2,3-bis(hydroxymethyl)-1,4-butanediol] produced by this strain. The *S. natalensis* npi287 PI factor mutant strain that cannot synthesize PI factor failed to produce pimaricin, thus explaining the role of PI factor in eliciting the biosynthesis of compound **55**. It has been shown that A-factor from *S. griseus* restored the pimaricin biosynthesis in PI factor mutant of *S. natalensis*, while conversely, PI factor did not elicit the production of streptomycin (**56**) in the A-factor mutant strain of *S. griseus*. This indicated that the activity of the PI factor was restricted to *S. natalensis*. Treatment of the wild type *S. natalensis* with pure PI factor resulted in an increase in the pimaricin

Table 2
Comprehensive list of secondary metabolites reported upon elicitation with cell-derived components.

Elicitor	Strain	Secondary metabolites induced	Mechanism of elicitation	References
N-acetylglucosamine [Under poor nutrient conditions]	*Streptomyces coelicolor*	Actinorhodin (**4**) Undecylprodigiosin (**5**)	Binding of glucosamine-6-phosphate to DasR led to de-repression in antibiotic synthesis	Ochi and Hosaka (2013), Rigali et al. (2008) and Zhu et al. (2014)
Unidentified fungal elicitor from *Penicillium chrysogenum* AS 3.5163	*S. natalensis* HW-2	Pimaricin (**55**)	Low molecular weight molecule from broth of *P. chrysogenum* AS 3.5163 displayed an elicitation effect	Wang et al. (2013)
N-butyryl-DL-homoserine lactone (HSL)	*Aspergillus fumigatus* MBC-F1-10 (P)	Emestrin A (**44**) Emestrin B (**45**)		Marmann et al. (2014) and Rateb et al. (2013)
PI-factor	*S. natalensis*	Pimaricin (**55**)	Hydrophilic auto inducer molecule regulated antibiotic production	Recio et al. (2004) and Zhu et al. (2014)
Glycerol 1,2 Propanediol 1,3 Propanediol ethylene glycol	*S. natalensis* npi287	Pimaricin (**55**)	Glycerol altered membrane permeability and led to internalization of the auto inducer PI-Factor	Recio et al. (2006) and Wang et al. (2013)
Oligosaccharides & polysaccharides	*S. rimosus* *Penicillium chrysogenum*	Unidentified secondary metabolites	Inhibition of Reactive Oxygen Species (ROS) led to overproduction of secondary metabolites	Radman et al. (2006)

production (Recio et al., 2004; Wang et al., 2013; Zhu et al., 2014). Glycerol elicited pimaricin production in the PI factor defective strain npi287 of *S. natalensis*. Alcohols 1,2 propanediol, 1,3 propanediol and ethylene glycol also had similar induction effect on pimaricin biosynthesis but their induction effects were lesser when compared with the effects of glycerol.



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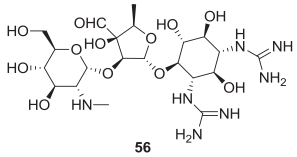

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A rapid elicitor screening platform in microbial and mammalian systems was developed based on the elicitor mediated inhibition of reactive oxygen species (ROS) and the subsequent overproduction of secondary metabolites (Radman et al., 2006). The effect of certain carbohydrate elicitors on overproduction of secondary metabolites was then assessed in *Streptomyces rimosus* M4018 and *P. chrysogenum* P2 strain ATCC 48271. ROS generation was evaluated using 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) as an indicator. Mannan oligosaccharides showed the most potent inhibition of ROS on all the systems assessed and overproduction of secondary metabolites as well. Thus chemical molecules repressing the ROS production in microbes could serve as elicitors of microbial secondary metabolites.

Antibiotics at sublethal concentrations can generate diverse metabolic responses in bacteria (Imai et al., 2015). Identification of elicitors that induce the expression of silent gene clusters under standard conditions is however a challenge. Recent work by Seyedsayamdost (2014) describes the development of a high throughput platform for the identification of elicitors of silent gene clusters in bacteria employing screening of small molecule libraries and genetic reporter fusions (e.g., *lacZ*/GFP), in order to assess the expression of the known cryptic gene cluster of interest. The elicitation effects of libraries composed of small molecules on two gene clusters (*mal* that is responsible for the expression of the virulence factor malleilactone and *bhc*, which is responsible for the expression of a histone deacetylase inhibitor) in *Burkholderia thailandensis* E264 were studied using *lacZ* fusions to putative genes in these clusters. Nine elicitors from a library of 640 compounds against the *mal* cluster and five elicitors from a library of 800 compounds against the *bhc* cluster were discovered. It was deduced that the majority of these antibiotics were active at sub-inhibitory/low concentrations. Thus with the aid of genetic manipulation encompassing reporter fusions, chemical molecule library platforms, and new mass spectrometric techniques it is now possible to identify elicitors for gene clusters of interest of, which product is either known or can be predicted by computational approaches.

3. Chemical elicitation

Chemical elicitation involves compounds of non-biological origin for elicitation process, which result in changes in the metabolomic profile. Chemical elicitors include inorganic compounds, rare earth elements, heavy metal ions, etc. The elicitation response depends on which/how many defense pathways are triggered and how strongly the pathways are activated. Table 3 shows a comprehensive list of chemical elicitors that have been shown to stimulate secondary metabolism in actinomycetes.

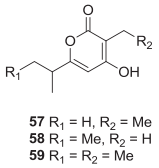
Screening of *Streptomycetes* with a Canadian compound collection encompassing 30,569 small molecules led to the identification of 19 compounds, which altered secondary metabolism (Craney et al., 2012). Four out of the nineteen compounds, called the "antibiotic remodeling compounds" (ARCs; ARC2, ARC3, ARC4, ARC5), induced actinorhodin (**4**) production in *S. coelicolor*. Treatment of *S. coelicolor* with the most active compound, ARC2, resulted in an elevation in the levels of the pyranones germicidins A–C (**57**–**59**) by three-fold and a retardation in the levels of prodiginines and daptomycin-like calcium dependent antibiotic CDA 4a (**60**) by *S. coelicolor*. LC–MS analysis revealed

Table 3
Comprehensive list of secondary metabolites reported upon chemical elicitation.

Elicitor	Strain	Secondary metabolites induced	Mechanism of elicitation	References
ARC2	*S. coelicolor*	Germicidins A–C (**57**–**59**), Actinorhodin (**4**)	Inhibition of fatty acid biosynthesis by inhibition of enoyl–acyl carrier protein reductase FabI	Craney et al. (2012), Ochi and Hosaka (2013), Ochi and Okamoto (2012) and Zhu et al. (2014)
	S. peucetius	Unknown metabolites		
Scandium (Sc^{3+})	*S. coelicolor*	Actinorhodin (**4**)	Upregulation of antibiotic activator transcripts and decrease in the bacterial alarmone ppGpp levels by binding of Scandium (Sc^{3+}) to ribosome alters antibiotic production	Kawai et al. (2007) and Ochi and Hosaka (2013)
	S. antibioticus & *S. parvulus*	Actinomycin D (**61**)		
	S. griseus	Streptomycin (**56**)		
	Bacillus subtilis 168	Bacilysin (**62**)		
Scandium and Lanthanum	*S. coelicolor* A3 (2)	Actinorhodin (**4**)	Nine genes upregulated, several products unidentified. Mechanism of action unknown	Ochi and Hosaka (2013), Ochi et al. (2014), Tanaka et al. (2010) and Zhu et al. (2014)
DMSO	*S. venezuelae* ATCC 10712	Chloramphenicol (**63**)	Effect at the translational level	Chen et al. (2000) and Pettit (2011)
	S. glaucescens	Tetracenomycin C (**64**)		
	S. azureus ATCC 14921	Thiostrepton (**65**)		
	S. lividans	Prodigiosin (**5**)		
Ethanol	*S. glaucescens*	Tetracenomycin C (**64**)	–	
Dimethyl sulphone	*S. venezuelae* ATCC 10712	Chloramphenicol (**63**)	–	
Ethanol	*S. venezuelae* ISP5230	Jadomycin B (**66**)	Induced either a heat shock response, or served as a metabolite precursor, or altered membrane permeability in *S. venezuelae* ISP5230	Doull et al. (1994) and Pettit (2011)
Sodium butyrate	*S. coelicolor*	Actinorhodin (**4**)	HDAC inhibitor	Moore et al. (2012) and Zhu et al. (2014)

– = Possible mechanism of elicitation not mentioned.

changes in the secondary metabolite profiles upon treatment with other actinomycetes strains like *Streptomyces pristinaespiralis*, *S. peucetius*, and *Kutzneria* sp. 744, indicating that the ARC2 mechanism is conserved among various actinomycetes. ARC2 showed structural synonymy with a fatty acid synthesis inhibitor and it was shown to affect secondary metabolite yields. It inhibited fatty acid biosynthesis through inhibition of enoyl–acyl carrier protein reductase FabI, an enzyme that catalyzes the rate limiting and final step in fatty acid biosynthesis. Fatty acid synthesis (primary metabolism) and polyketide synthesis (secondary metabolism) share acyl-CoA precursors (malonyl-CoA & acetyl-CoA). Thus ARC2 might act through the partial inhibition of enoyl–acyl carrier protein reductase FabI leading to a halt in the primary metabolism and diverting at the same time the precursor flow towards antibiotic production. ARC2 treatment also induced the expression of unknown metabolites in *S. peucetius*, thus explaining the role of the elicitor in the discovery of new metabolites (Craney et al., 2012; Ochi and Hosaka, 2013; Zhu et al., 2014). Thus reconfiguring the metabolism by small molecules like ARC2 and triclosan that inhibit fatty acid biosynthesis or primary metabolism is an effective approach in enhancing polyketide synthesis and activating silent polyketide gene clusters in actinomycetes.



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58 R_1 = Me, R_2 = H
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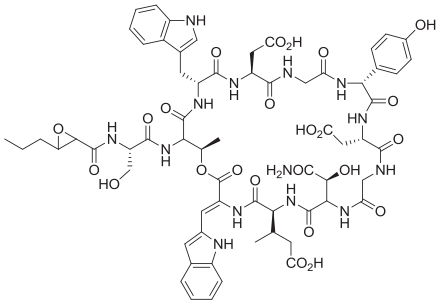

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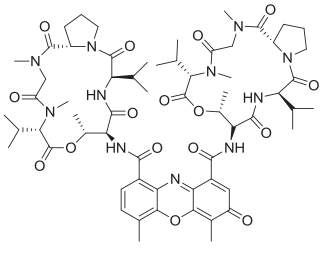
Elicitation effects were observed on the antibiotics actinorhodin (**4**), dactinomycin (**61**), streptomycin (**56**), and bacilysin (**62**) production by *S. coelicolor*, *Streptomyces antibioticus*, and *Streptomyces parvulus*, *S. griseus* and *B. subtilis* 168 upon treatment with the rare earth element (REE) scandium (Sc^{3+}). Other REEs like Yttrium (Y), Lanthanum (La), Cerium (Ce), and Europium (Eu) also upregulated actinorhodin production in *S. coelicolor*. Scandium (Sc^{3+}) enhanced the levels of antibiotic production by these strains by multiple-fold when compared to the untreated sets. Pathway-specific antibiotic activator (*actII-ORF4*) transcripts were upregulated in REE treated groups indicating the activation of antibiotic production. The biosynthetic gene cluster for actinorhodin production, which remained silent in *S. lividans* is highly expressed upon treatment with Sc^{3+}. This throws light on the applicability of using REEs for expression of cryptic gene clusters. Although the exact sites of action of REEs are unknown, it is thought that they might bind to ribosomes (Kawai et al., 2007; Ochi et al., 2014; Zhu et al., 2014). A study by Tanaka et al. (2010) showed that REEs (except promethium (Pm)) enhanced the production of compound **4** in *S. coelicolor* A3(2). Of these, the most effective ones were Sc^{3+} and La^{3+}, which altered the transcription of 17 genes belonging to 17 secondary metabolite biosynthetic gene clusters in *S. coelicolor* (Ochi and Hosaka, 2013; Ochi et al., 2014; Tanaka et al., 2010).



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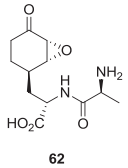
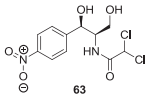


62

Supplementation of fermentation media with the solvent dimethylsulfoxide (DMSO) elevated the levels of metabolites from different biosynthetic families in *Streptomyces* sp. Three-fold increases were achieved in the production of chloramphenicol (**63**) by *S. venezuelae* ATCC 10712 and the polyketide tetracenomycin C (**64**) by *Streptomyces glaucescens*. A two-fold increase was observed in production of the peptide thiostrepton (**65**) by *Streptomyces azureus* ATCC 14921 upon supplementation with 3% (v/v) DMSO. DMSO has also been shown to enhance the levels of prodigiosin (**5**) production by *S. lividans*. LC–MS analysis revealed an alteration in the metabolite profiles of the antibiotic producing *Bacillus circulans* and *Bacillus polymyxa* upon treatment with DMSO, while ethanol enhanced the production of tetracenomycin C by *S. glaucescens* but repressed chloramphenicol production in *S. venezuelae*. Chloramphenicol production was increased in the presence of dimethylsulfone (DMSN) but to a lesser extent than that with DMSO (Chen et al., 2000; Pettit, 2011).



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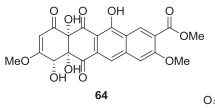

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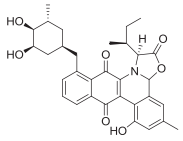

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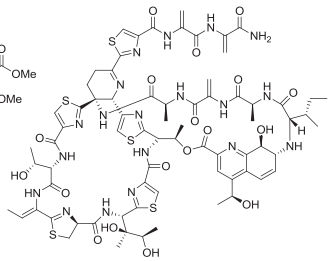


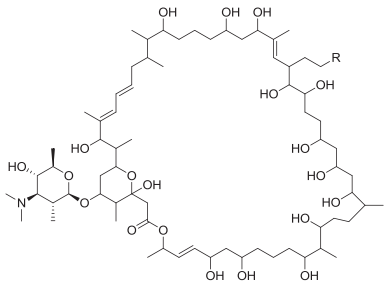
65

Production of the pigmented benzoxazolophenanthridine antibiotic, jadomycin B (**66**), in *S. venezuelae* ISP5230 in D-galactose-L-isoleucine production medium was increased upon addition of ethanol. It was speculated that ethanol either induces a heat shock response in *S. venezuelae* ISP5230, serving as a metabolite precursor, or alters membrane permeability (Doull et al., 1994; Pettit, 2011).

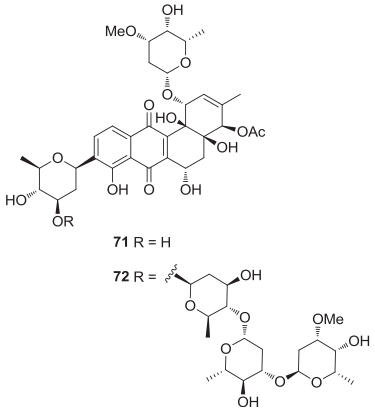
4. Molecular elicitation

Apart from the cultivation-dependent elicitation approaches mentioned above, the methods for identifying the metabolic products from silent gene clusters of microbes identified through genomic mining has been reviewed (Challis, 2008). Mining microbial genomes for identifying silent gene clusters and the subsequent characterization of the cryptic products through gene-knock out studies coupled with analytical techniques like HPLC–MS and MALDI-TOF imaging, provides another platform to excavate the hidden potential of actinomycetes (Müller and Wink, 2014).

New natural products encoded by silent gene clusters could be unmasked by constitutive gene expression of putative regulators present in the same gene cluster. For instance, constitutive overexpression of a pathway specific regulator protein (similar to Large ATP binding of the LuxR, LAL) family that belonged to the cryptic type I polyketide synthase (PKS) gene cluster in *Streptomyces ambofaciens* ATCC 23877 directed the expression of four 51-membered glycosylated macrolides, stambomycins A–D (**67**–**70**). Stambomycins A–D (**67**–**70**) showed moderate antibacterial activity against Gram-positive bacteria and potent anti-proliferative activities against human cell lines (Laureti et al., 2011; Ochi and Hosaka, 2013; Ochi et al., 2014; Zhu et al., 2014).

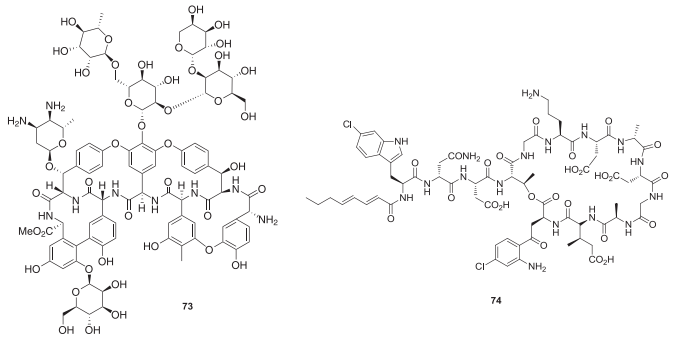


With the assistance of new bioinformatics tools, it is now possible to deduce the natural products encoded from the orphan and silent gene clusters; thus linking genes to the molecules that they encode in-silico. Furthermore, applications of genetic engineering and analytical tools to these cryptic gene clusters could validate the in-silico predictions and characterize their products in-vitro. Sequencing the genome of *Streptomyces chattanoogensis* L10 led to identification of twelve PKS gene clusters. The metabolic products of only two of these clusters have been identified. An angucycline-like antibiotic, chattamycin biosynthetic gene cluster was identified to be silent in *S. chattanoogensis* L10 and it was subsequently activated by overexpression of the gene *chaI* encoding the putative pathway-specific activator. This led to the identification of the angucycline antibiotics, chattamycins A and B (**71**–**72**) (Zhou et al., 2015).

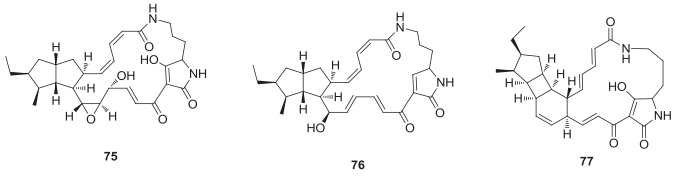


Twenty-nine cryptic gene clusters were identified in *Amycolatopsis japonicum* using genome mining and one among these was found to be a putative glycopeptide gene cluster. With the aid of bioinformatic tools, ristomycin A (**73**), an antibacterial and a diagnosis compound (used to detect hereditary diseases) was postulated to be the possible product of this cluster. Genetic manipulation of *A. japonicum* was done by introducing the *bbr* gene (encoding the transcriptional activator of the glyopeptide balhimycin biosynthesis) from *Amycolatopsis balhimycina* (*bbr*Aba) into *A. japonicum*. Using LC-MS, MS/MS and NMR, it was confirmed that genetically engineered *A. japonicum*/pRM4-*bbr*Aba produced ristomycin A (**73**) due to the activation of cryptic ristomycin gene cluster (Spohn et al., 2014). Thus genome mining combined with genetic manipulation techniques could activate cryptic gene clusters in actinomycetes.

Bioinformatic analysis of the draft genome sequence of the marine actinomycete *Saccharomonospora* sp. CNQ490 revealed the diverse metabolic potential of this strain. A 67-kb (nonribosomal peptide synthetase (NRPS)) *tar* gene cluster encoding taromycin A (**74**) was identified to be silent in this strain. Transformation-associated recombination cloning of this orphan biosynthetic gene cluster led to the subsequent activation of the gene cluster with the production of dichlorinated lipopeptide antibiotic taromycin A (**74**). Thus direct cloning and efficient 'plug-and-play' approaches could uncover the vast hidden genomic potential in actinomycetes (Yamanaka et al., 2014).



Synthetic biology is an emerging strategy to awaken the cryptic metabolic gene clusters in actinomycetes. In this strategy, the identified silent biosynthetic gene clusters are subjected to PCR-amplification or chemical synthesis followed by gene cluster reconstruction by selecting suitable heterologous promoters. Once reconstructed, these gene clusters are assembled in yeast and the assembled constructs isolated from yeast are then re-transformed in *E. coli* and transferred to a heterologous host after verification of the assembled constructs. Application of analytical techniques like LC–MS, MS/MS and NMR aids in detection and characterization of the products from the heterologous host. Employing this synthetic biology strategy led to the activation of cryptic polycyclic tetramate macrolactam (PTM) biosynthetic gene cluster SGR810-815 in *S. griseus* along with the discovery of three new PTMs **75**–**77** (Luo et al., 2013). Using a similar synthetic biology approach, the silent spectinabilin pathway in *Streptomyces orinoci* was awakened (Shao et al., 2013).



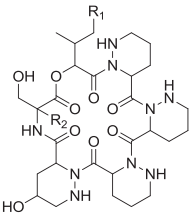
Ribosome engineering is a strain-improvement strategy adopted to enhance the production of antibiotics by regulating the ribosomal components (either the ribosomal proteins or rRNA). This involves introduction of multiple drug resistant mutations in bacteria (since, majority of the antibiotics target ribosomes) leading to high levels of antibiotic production, which are predominantly due to the acquisition of mutant ribosomes and ppGpp (Guanosine tetraphosphate) synthesis activity, which is a key mediator when bacteria encounter adverse environmental conditions such as the limited availability of an essential nutrient (Wang et al., 2008). Drug-resistant bacterial mutants isolated from soil have been shown to synthesize antibacterial compounds piperidamycins A, D, and F (**78**–**80**), which were lacking in the wild type. Activation of this class of compounds was observed in 6% of the non-*Streptomyces* sp. and 43% of the *Streptomyces* sp. among the 1068 soil-derived actinomycetes. Interestingly, all of the antibacterial-producing, drug-resistant actinomycete mutants carried mutations in the genes encoding RNA polymerase and/or the ribosomal protein S12. Thus selection of drug resistant actinomycete mutants could allow the discovery of new classes of antibacterial compounds (Hosaka et al., 2009; Ochi and Hosaka, 2013).

Tanaka et al. used rifampin resistance (*rpoB*) mutations to activate cryptic or poorly expressed secondary metabolite biosynthetic gene clusters in various actinomycetes (Tanaka et al., 2013). The insertion of certain *rpoB* mutations effectively increased antibiotic production in

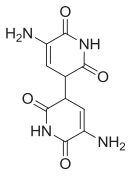
S. griseus (streptomycin producer), *S. coelicolor* (actinorhodin producer), *S. antibioticus* (actinomycin producer), *Streptomyces lavendulae* (formycin producer), *Saccharopolyspora erythraea* (erythromycin producer), and *Amycolatopsis orientalis* (vancomycin producer).

Histone deacetylases (HDAC) alter chromatin structure and thus influence the expression of biosynthetic gene clusters. Analysis for presence of histone deacetylase genes in *S. coelicolor* revealed the existence of three HDAC-like genes. Evaluation on the effect of the HDAC inhibitor, sodium butyrate, on antibiotic production in *S. coelicolor* demonstrated a contrasting influence on actinorhoidin (**4**) production. While sodium butyrate, similar to GlcNAc, upregulated the production of compound **4** under poor nutrient conditions, an opposite effect was observed under nutrient-rich conditions (Moore et al., 2012; Zhu et al., 2014). The analogous effects of GlcNAc and sodium butyrate on actinorhodin production suggest a common elicitation mechanism, which is yet to be explored.

Olano et al. used three different genome mining-based approaches to activate the expression of the silent gene clusters in *S. albus* J1074, which is not known to produce any bioactive natural product under standard growth conditions (Olano et al., 2014). After insertion of a strong and constitutive promoter such as *ermE**p (promoter of the erythromycin resistance genes) in the front of selected genes of two clusters, production of the blue pigment indigoidine (**81**) and of two novel members of the polycyclic tetramate macrolactam family 6-*epi*-alteramides A and B (**82** and **83**) was activated. Overexpression of positive regulatory genes from *S. albus* J1074 also activated the biosynthesis of compounds **82** and **83** and heterologous expression of the regulatory gene *pimM* (regulatory gene of the pimaricin pathway in *S. natalensis*) activated the simultaneous production of candicidins and antimycins.



78 R_1 = H, R_2 = H
79 R_1 = H, R_2 = Me
80 R_1 = Me, R_2 = Me



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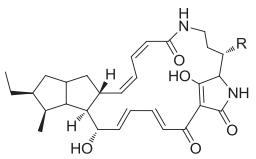
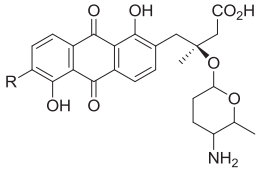
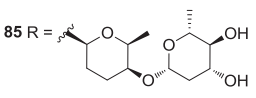


82 R = OH
83 R = H

A simple and effective method called reporter-guided mutant selection (RGMS) was developed by Guo et al. (2015). This method makes use of genome scale random mutagenesis for activation of silent gene clusters and a promoter–reporter system for identification of the desired mutants based on specific phenotypes, indicating the activation of transcription of the silent genes. RGMS re-activated the jadomycin (**66**) production in *S. venezuelae* ISP5230 and also activated the silent *pga* gene cluster in *Streptomyces* sp. PGA64 leading to the production of the anthraquinone aminoglycosides gaudimycins D and E (**84**–**85**). Thus such platforms that might generate mutations for activating the cryptic gene clusters coupled with specific reporter systems might identify and characterize the products of the silent gene clusters.



84 R = H



85 R =

From genome sequencing data of actinomycetes, it is evident that those genomes encode a large potential of future drug leads. Classification of gene clusters into families (GCFs) based on the available sequence information and MS data provides an avenue for de novo correlation of natural products and their corresponding biosynthetic genes. A systematic bioinformatics framework was reported, which could be used to study natural product gene clusters. Out of 830 bacterial genome sequences, 11,422 gene clusters were assigned into 4122 GCFs and validated with MS data. Further, the assigned and unassigned GCFs were linked to known natural products. A method of this type provides a roadmap to access the complete diversity of natural product gene clusters in a systematic way (Doroghazi et al., 2014).

Genomic approaches coupled with metabolomics could prevent the discovery of known metabolites from actinomycetes with the aid of specific de-replication strategies (Cimermancic et al., 2014; Harvey et al., 2015; Gaudencio and Pereira, 2015; Letzel et al., 2013; Müller and Wink, 2014; Tawfike et al., 2013). Using metabolomics, spectral data from the samples could be processed by differential expression analysis using software such as MZmine (Pluskal et al., 2010), MZmatch (Pluskal et al., 2012) and XCMS (Tautenhahn et al., 2012) coupled with databases such as Dictionary of Natural Products (Buckingham J. Dictionary of Natural Products. Version 20:2. London, UK, Chapman and Hall/CRC, 2012), AntiBase (Laatsch H. Antibase Version 4.0 — The Natural Compound Identifier. Wiley-VCH Verlag GmbH & Co. KGaA, 2012) or MarinLit (Blunt J. MarinLit. University of Canterbury, New Zealand, 2012) to differentiate known compounds against novel ones and LC-1D/2D NMR data could help in confirmation of these dereplication results (Dashti et al., 2014; Grkovic et al., 2014). Subjecting the processed data generated by this way to principle component analysis (PCA) and or orthogonal partial least squares discriminant analysis (OPLS-DA) generates S-plots and heat maps (Krug and Müller, 2014; Macintyre et al., 2014). Finally, with pattern recognition it is possible to define novel natural products that could be further subjected to scale-up efforts (Harvey et al., 2015; Krug and Müller, 2014). Chemical structures of these natural products from the scale-up fermentation studies could then be elucidated with analytical LC–MS-NMR spectral data.

5. Conclusions and outlook

Actinomycetes are rich resources for natural products with discrete pharmaceutical and biotechnological applications. The rapidly growing number of actinomycete genome sequences reveals their potential for biosynthesizing a plethora of secondary metabolites that is much higher than the actual number of compounds produced during classical fermentation. Subjecting the actinomycetes to molecular, biological, or chemical elicitation is an effective strategy to provoke the expression of unexpressed or poorly expressed bioactive metabolites and further, diversification of secondary metabolite profiles, thereby supplying novel drug leads to the natural products based pharmaceutical pipeline. However, the lack of information on the precise physiological and/or ecological signals to stimulate the biosynthetic machinery makes the research on activating silent gene clusters a challenging task, which requires interdisciplinary strategies to activate them and to explore their full chemical diversity. This starts with the application of genome mining with the help of bioinformatic algorithms such as antiSMASH (Blin et al., 2013) and NapDoS (Ziemert et al., 2012) to identify the putative silent gene clusters. The right elicitation approach to activate those cryptic pathways could be then identified using high throughput platform (Seyedsayamdost, 2014; Zazopoulos et al., 2003). Efficient dereplication tools such as metabolomics and molecular networking are then needed to avoid isolation of known compounds and to evaluate the possible novelty of the induced metabolites. Finally, structural identification of the new cryptic products through LC–MS, MS/MS and NMR will guide to the novel chemical scaffolds (Doroghazi et al., 2014; Harvey et al., 2015; Kersten et al., 2011; Krug and Müller, 2014). Besides, we posit that the natural

biodiversity is far from being uncovered and that natural collections will continue to hold promise for the future. Most of the elicitation and mixed fermentation studies were carried out on actinomycetes from terrestrial soils. The marine environment in particular offers novel and chemically rich actinomycete species, which is still underexplored in terms of elicitation and co-cultivation. Furthermore, challenging metabolically talented microorganisms with various elicitors might be a successful approach to identify chemical novelty. The required conceptual advancement would be aided by the directed and coordinated datasets and that will ultimately reveal the global regulatory networks (i.e., stress, starvation, quorum sensing) into, which secondary metabolism is embedded. A multidisciplinary approach gathering the knowledge of molecular biologists, natural products chemists, and microbiologists will enhance the elicitation pipeline towards production of valuable natural products for pharmaceutical applications. Thus the identification of cryptic gene clusters by correlating genome mining and gene expression analyses with elicitation approaches will provide a new avenue to the treasure trove of natural products from actinomycetes.

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