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REVIEW

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Elicitation for activation of the actinomycete genome's cryptic secondary metabolite gene clusters

Seham S. El-Hawary,†^a Marwa H. A. Hassan, ^[b]†^b Ahmed O. Hudhud,^c Usama Ramadan Abdelmohsen (1) *de and Rabab Mohammed*b

This review summarizes the recent advances in the elicitation approaches used to activate the actinomycete genome's cryptic secondary metabolite gene clusters and shows the diversity of natural products obtained by various elicitation methods up to June 2022, such as co-cultivation of actinomycetes with actinomycetes, other non-actinomycete bacteria, fungi, cell-derived components, and/or algae. Chemical elicitation and molecular elicitation as transcription factor decoys, engineering regulatory genes, the promoter replacement strategy, global regulatory genes, and reporter-guided mutant selection were also reported. For researchers interested in this field, this review serves as a valuable resource for the latest studies and references

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Introduction 1.

Regular protocols used for the discovery of bacterial-based natural products often involve microorganism fermentation on different media followed by chemical and biological screening of the extracts, seeking any interesting bioactive compounds. The advent of genome-sequencing technology has enabled us to scan the substantial amount of published genome data for gene clusters encrypting the previously recognized natural products, bringing in a second golden age in drug discovery. Recently, numerous new approaches were published unlocking bacterial cryptic pathways, including studies that focus on raising transcription levels and enlivening dead genes.1-3 Actinomycetes are unicellular, Gram-positive filamentous bacteria, belonging to the order Actinomycetales. They are broadly dispersed in nature and can be found in diverse habitats across the world. Actinomycetes are notable for producing a diverse variety of natural products with a wide range of biological activities and structurally diverse specialized metabolites.1 Over 200 drugs based on metabolites isolated from actinomycetes are currently in clinical trials or have been approved by the FDA for the treatment of infections, immune

system abnormalities and cancer chemotherapeutic and to treat emerging health threats.² Under conventional culture conditions, the majority of bioactive metabolites encrypted in actinomycete genomes are not activated and tendency to rediscover known secondary metabolites increases.3 Therefore, various approaches has been devoted to eliciting the underlying gene clusters in order to discover and isolate novel lead natural products and molecular scaffolds;4-6 such as: mixed fermentation or co-cultivation,7 fermentation conditions alteration which known as "one strain many compounds" (OSMAC)8 and eliciting the bacterial cells with external signals either chemically or biologically.9,10 This review summarizes notable and successful examples of elicitation methods based on literature search from 2015 until June 2022, including changing culture conditions, modifying the medium, co-cultivating with different strains, and adding a biosynthetic precursors or epigenetic modifiers (Fig. 1). This review is complementary to the first part that was previously published.11

2. Biological elicitation

Biological elicitation is one of the most actively pursued techniques to trigger the synthesis of natural products in actinomycetes.

2.1. By microbial co-cultivation

The confrontation of microorganisms in various habitat may activate cryptic gene clusters and trigger silent pathways to produce new secondary metabolites. These outcomes are based on the effective interactions between co-cultivated microorganisms.12 For example, horizontal gene transfer, physical cell-

^aDepartment of Pharmacognosy, Faculty of Pharmacy, Cairo University, Cairo, Egypt ^bDepartment of Pharmacognosy, Faculty of Pharmacy, Beni-Suef University, Beni-Suef 62511, Egypt. E-mail: rmwork06@yahoo.com; rababmohammed@bsu.pharm.edu.eg ^cDepartment of Pharmacognosy, Faculty of Pharmacy, Merit University, Sohag 82511, Egypt

^dDepartment of Pharmacognosy, Faculty of Pharmacy, Minia University, Minia 61519, Egypt. E-mail: usama.ramadan@mu.edu.eg

^eDepartment of Pharmacognosy, Faculty of Pharmacy, Deraya University, New Minia 61111, Egypt

[†] These authors contributed equally to this work.



Fig. 1 Graph representing the percentage of application of different elicitation methods.

cell interactions and production of enzymes or quorum sensing molecules.¹¹ Co-cultivation-based elicitation can thus lead to the generation of molecules that are not synthesized in monoculture, thus aiding in the discovery of cryptic and weakly expressed secondary metabolites. However, finding suitable partners for co-cultivation remains challenging. Co-cultivation can be further categorized as (a) co-cultivation of actinomycetes with other actinomycetes, (b) co-cultivation of actinomycetes with other non-actinomycete bacteria, and (c) cocultivation of actinomycetes with fungi. The following section describe the process, the differences and the results on each of the three categories mentioned above.

2.1.1. Co-cultivation of actinomycetes with actinomycetes (Table 1 and Fig. 2). Co-culture of two actinobacteria from the Red Sea, *Rhodococcus* sp. UR59 and *Actinokineospora spheciospongiae* strain EG49 revealed stimulation of several metabolites production which were not observed in their axenic cultures. Using biologically guided approach, angucyclines; actinosporins E (1), H (2), G (3), tetragulol (4) and the anthraquinone, capillasterquinone B (5) were isolated and identified. The isolated metabolites showed *in vitro* antimalarial activity against *Plasmodium falciparum*.¹³

Co-cultivation of two actinomycetes associated with marine sponge, *Actinokineospora* sp. EG49 and *Micromonospora* sp. UR56 resulted in the accumulation of five metabolites that were not traced in their independent cultures. These metabolites were isolated and identified as dimethyl phenazine-1,6-dicarboxylate (6), phencomycin (7), tubermycin (8), *N*-(2-hydroxyphenyl)-acetamide (9), *p*-anisamide (10). Compounds 6–8 and 10 showed high antibacterial, antibiofilm and cytotoxic activities.¹⁴

A change in natural product biosynthesis occurred in *Micromonospora* sp. UA17 isolated from the Red Sea Sponge *Coscinoderma mathewsi*, when co-cultivated with the mycolic acid containing *Nocardia* sp. UA 23 and *Gordonia* sp. UA19. Chlorocardicin (**11**), neocopiamycin A (**12**), and chicamycin B (**13**) were identified in the co-culture extracts, but not in the corresponding monocultures. This suggests a role for mycolic

acid in the stimulation of encoded natural product biosynthesis pathways. Co-culture extracts showed antibacterial, antiparasitic and antifungal activities higher than monocultures.¹⁵ *Streptomyces* sp. UR23 co-fermentation with a strain containing mycolic acid *Nocardia* sp. UR27 stimulating the production of a possible antitrypanosomal agents bafilomycin D (14) and bafilomycin A1 (15).¹⁶ In addition, three new compounds identified as mirilactams (C–E) (16–18) were isolated in the coculture of *Actinosynnema mirum* NBRC 14064 with *Tsukamurella pulmonis* TP-B0596.¹⁷

Nguyen and his colleagues found that the butenolide compounds in *Streptomyces albus* J1074 stimulated the production of avermetin (**19**) in *Streptomyces avermitilis* in an *in vivo* metabolic profiling assay with imaging mass spectrometry (IMS). This demonstrated the complex chemical interactions in streptomycetes through interspecies signals.¹⁸

Furthermore, the combined culture of a *Dietzia* sp. UR66 with *Saccharomonospora* sp. UR22 resulted in the production of a novel brominated oxo-indole alkaloid Saccharomonosporine A (**20**), convolutamydine F (**21**) and (S) 6-bromo-3-hydroxy-3-(1*H*-indol-3-yl) indolin-2-one (**22**) with a promising Pim-1 kinase inhibitors that mediates tumor cell growth inhibitory effect, along with other known induced metabolites; (S) 6-bromo-3-hydroxy-3-(1*H*-indol-3-yl) indolin-2-one (**22**) and nonactin (**23**).¹⁹ Amycomicin (**24**), a modified fatty acid with an epoxide isonitrile head, was discovered to be a selective and potent inhibitor of *Staphylococcus aureus* after co-culture with *Amycolatopsis* sp. AA4 as the producing strain and *Streptomyces coelicolor* M145 as the inducing strain.²⁰

In the co-culture broth of *Tsukamurella pulmonis* TPB0596 with a rare actinomycete *Micromonospora wenchangensis* HEK-797, only two new macrolactams (dracolactams A and B) were detected, whereas the axenic medium of strain HEK-797 yielded a 26-membered polyene macrolactam, which was probably the precursor of dracolactams A (**25**) and B (**26**).²¹

From another co-culture of *Rhodococcus* sp. WMMA-185 and *Micromonospora* sp. WMMB-235, a two-marine invertebrate-associated bacteria, a novel antibiotic, keyicin (27), was

Table 1 Comprehensive list of secondary metabolites reported upon co-cultivation of actinomycetes with actinomycetes

Interaction partners	Induced secondary	Metabolites activity	Reference
Actinokineospora spheciospongiae strain EG49	Actinosporins E (1)	Anti-malarial	13
and <i>Rhodococcus</i> sp. UR59	Actinosporins H (2)		
	Actinosporins G (3)		
	Capillasterquipope P (5)		
Micromonospora sp. LIR56 and	1 6-Dicarboxylate (6)	Antibacterial	14
Actinokineospora sp. EG49	Phencomycin (7)	minouccentar	11
Γ_{r}	Tubermycin (8)	Cytotoxic antibiofilm	
	<i>N</i> -(2-hydroxyphenyl)-acetamide (9) <i>p</i> - anisamide (10)		
Micromonospora sp. UA17, Gordonia sp.	Chlorocardicin (11)	Antibacterial	15
UA19, and <i>Nocardia</i> sp. UA 23	Neocopiamycin A (12)	Antifungal	
	Chicamycin B (13)	Antiparasitic	
Streptomyces sp. UR23 and Nocardia sp.	Bafilomycin D (14)	Antitrypanosomal agents	16
UR27	Bafilomycin A1 (15)		
Actinosynnema mirum NBRC 14064 Tsukamurella pulmonis TP-B0596	Mirilactams C–E, 1–3 (16, 17, 18)	Macrolactams	17
Streptomyces avermitilis and Streptomyces albus J1074	Avermectin (19)	Pesticides	18
Saccharomonospora sp. UR22 and Dietzia sp.	Saccharomonosporine A (20) Convolutamydine F (21)	Antiproliferative activity	19
	(S) 6-Bromo-3-hydroxy-3-(1 <i>H</i> -indol-3-		
Strontonning and inclose M145	yi) indoin-2-one (22)	Antibiotic activity	20
Armicolatonsis sp. AAA	Amycomycin (24)	Antibiotic activity	20
Amycolalopsis sp. AA4 Micromonospora wenchangensis HEK-797	Dracolactams A(25)	Antifungal antibiotics	21
Tsukamurella nulmonis TPB0596	Dracolactams B (26)	Antifulgar antibiotics	21
Rhodococcus sp. WMMA-185	Kevicin (27)	Selectively active against gram-	22
Micromonospora sp. WMMB-235		positive bacteria	
Cutibacterium avidum	Propionate (28)	It is a conjugate base of	23
Bifidobacterium longum subsp. infantis and		a propionic acid	
Bifidobacterium bifidum			
Streptomyces nigrescens HEK616	5-Alkyl-1,2,3,4-tetrahydroquinolines	Novel antifungals	24
Tsukamurella pulmonis TP-B0596	(29)	Description of the state of the state	25
Streptomyces nigrescens HEK616	[5,5]-Spirohemiaminals (30)	Broad antimicrobial activities	25
Strentomyces sp. ANAM-5 and AIAH-10	Ethyl acetate extract	Antimicrobial and anticoncer	26
Sucpromyers sp. Analyr's and Alan-10	Emplateate chuaet	activities	20
Streptomyces tendae KMC006	Gordonic acid (31)	Weak antibacterial activity	27
Gordonia sp. KMC005	()		
Streptomyces sp. NZ-6	Niizalactams A–C (32–34)	Antifungal antibiotic	28
Tsukamurella pulmonis TP-B0596		-	
Streptomyces cinnamoneus NBRC 13823	Arcyriaflavin E (35)	Cytotoxic	29
Tsukamurella pulmonis			
Streptomyces sp. CJ-5	Chojalactones A–C (36–37)	Cytotoxic	30
Tsukamurella pulmonis TP-B0596	(22)		21
Streptomyces Itviaans TK23/pGSBC1	Goadsporin C (38)	Antiblotic	31
Isukumurenu purmonis IP-B0596 Catenulonlanes sp. BD067221	Catenulobacting A and P (20, 40)	Sidernhore	30
Tsukamurella nulmonis TP-R0596	Catchulobacuns A and D (39, 40)	siderphote	34
Umezawaea sp. RD066910	Umezawamides A and B (41, 42)	Anti-leukemia and antifungal	33
Tsukamurella pulmonis TP-B0596		activity	
Nocardiopsis sp. FU40 (ΔApoS)	Ciromicin A (43)	Cytotoxicity (acute myelogenous	34
Phodococcus surgicicaniancia	Circomiain P (44)	cutotoviaitų (store lika muslaid	25
Knouococcus wratisiaviensis	Chomicin B (44)	progenitor cells)	33

isolated. It had specific inhibitory effect against Gram-positive bacteria Methicillin Sensitive *Staphylococcus aureus* (MSSA) and *B. subtilis*, with MIC values of 2.5 μ M and 9.9 μ M, respectively. Unlike many anthracyclines, keyicin may alter fatty acid

metabolism and showing antibacterial activity without causing nucleic acid damage.²²

Cutibacterium avidum growth and production of propionate (28) enhanced by co-cultivation with *Bifidobacterium longum*



Fig. 2 Chemical structures of compounds 1–44 resulted from the cocultivation of actinomycetes with actinomycetes. The configuration of all stereocenters in each compound is shown as given in the literature.

subsp. infantis and *Bifidobacterium bifidum* which confirmed the metabolic cross-feeding strategy.²³

2.1.2. Co-cultivation of actinomycetes with other nonactinomycete bacteria (Table 2 and Fig. 3). Interspecies interaction between organisms living in the same environment is thought to trigger the structural variety of specialized metabolites.¹¹ The co-fermentation of marine strain *Streptomyces* sp. CGMCC4.7185 and *Bacillus mycoides* obtained from Nanji Island marine sediments was the first report of using a microorganism co-culture system to increase the yields of five known tryptamine derivatives. The average yields of the isolated compounds *N*-acetyltryptamine (45), *N*-propanoyltryptamine (46), bacillamide A (47), bacillamide B (48) and bacillamide C (49) were 14.9, 2.8, 3.0, 13.7 and 9.6 mg L⁻¹, respectively, that was better than that produced by the axenic culture.³⁶

Liang *et al.* stated that co-cultivation method induced more mass characteristics than the heat-killed inducer cultures, and both approaches resulted in the initiation of major characteristics not seen with any other induction method. *N*-Carbamoyl-2-hydroxy-3-methoxybenzamide (**50**) and carbazoquinocin G (**51**), two new secondary metabolites produced through co-culturing of producer *Streptomyces* sp. RKND-216 with inducers *Alteromonas* sp. RKMC-009 and *M. smegmatis* ATCC 120515, respectively.³⁷

Dentigerumycin E (52), a new piperazic acid-bearing cyclic peptide isolated from the co-culture of marine *Streptomyces albogriseolus* strain B24 and *Bacillus cereus* and shown antimetastatic and antiproliferative activities against: liver, lung, stomach, breast and colorectal cancer.³⁸

Co-cultivation of *Streptomyces* sp. PTY087I2 with human pathogens such as: *Bacillus subtilis, Pseudomonas aeruginosa,* methicillin-sensitive and methicillin-resistant *Staphylococcus aureus*, resulted in increased production of the three antibiotics, granaticin (53), granatomycin D (54), and dihydrogranaticin B (55). Furthermore, biological activities against the Gram-positive human pathogens used in these study was significantly increased.³⁹ Increasing the use of co-culture studies to enable competitive interactions could boost metabolite synthesis and help to learn more about these microbial relationships.

2.1.3. Co-cultivation of actinomycetes with fungi (Table 3 and Fig. 4). The co-culture of *Aspergillus niger* and *Mycobacterium smegmatis* led to the production of a pigment by *A. niger* as well as an enhance in the extract's cytotoxic activity against human prostate cancer cells. An examination of natural products in the co-culture broth extract revealed that the increase in cytotoxic activity was driven by the development of malformin C (60), as well as the production of TMC-256A1, desmethylkotanin, and aurasperone C under co-culture conditions.⁴⁴

In *Streptomyces natalensis* HW-2 fermentation, a fungal elicitor from *Penicillium chrysogenum* AS 3.5163 showed an inductive effect on the production of natamycin (**61**). It was first observed that the fungal elicitor altered transcriptional levels in *S. natalensis* HW-2. The main conclusion is that the fungal elicitor increases the amount of precursor and changes the expression of natamycin related genes and secondary metabolic regulators.⁴⁵

Co-cultivation of *Bionectria* sp., obtained from the seeds of the tropical plant *Raphia taedigera* with *Streptomyces lividans* Table 2 Comprehensive list of secondary metabolites reported upon co-cultivation of actinomycetes with other non-actinomycete bacteria

Interaction partners	Induced secondary	Metabolites activity	Reference
Streptomyces sp. CGMCC4.7185	Tryptamine (45 , 46)	Algicidal	36
Bacillus mycoides	Bacillamides (47, 48, 49)	C C	
Streptomyces sp. RKND-216	N-Carbamoyl-2-hydroxy-3-	Antimicrobial	37
Alteromonas sp. RKMC-009	methoxybenzamide (50)	Cytotoxic activity	
Streptomyces sp. RKND-216	Carbazoquinocin G (51)	Antimicrobial	37
M. smegmatis ATCC 120515		Cytotoxic activity	
Streptomyces albogriseolus strain	Dentigerumycin E (52)	Antiproliferative	38
B24			
Bacillus cereus		Antimetastatic	
Streptomyces sp. Strain PTY08712	Granaticin (53)	Increased antibacterial activity	39
methicillin-resistant Staphylococcus	Granatomycin D (54)		
aureus (MRSA)	Dihydrogranaticin B (55)		
Streptomyces coelicolor	Actinorhodin (56)	A benzoisochromanequinone dimer	40
Myxococcus xanthus DK1622		polyketide antibiotic	
Streptomyces coelicolor A3	Undecylprodigiosin (57)	Immunosuppressive	41
E. coli C600		Antitumor	
Streptomyces sp. MA37	Be-13793c (58)	Anti-cancer activity	42
Pseudomonas sp.			
Streptosporangium sp. KDCAGE35	Funisamine (59)		43

yielded two novel *o*-aminobenzoic acid derivatives, bionectriamines A (**62**) and B (**63**), along with two other previously reported tris(2,4-di-*tert*-butylphenyl) phosphate (**64**) and 6,8dihydroxyisocoumarin-3-carboxylic acid (**65**). None of the reported compounds were found in axenic fungal or bacterial cultures, showing that co-cultivation with bacteria activated silent biogenetic gene clusters.⁴⁶ Amphotericin B (**66**), a wellknown antifungal compound resulted from the co-cultivation of *Streptomyces albospinus* RLe7 and *Coniochaeta* sp. FLe4, endophytes isolated from the roots of the Brazilian medicinal plant *Lychnophora ericoides*. Amphotericin B was identified as one of the substances responsible for antifungal action as well as the induction of a red-pigmented fungal phenotype.⁴⁷

A series of cytochalasans (rosellichalasin (67) and five aspochalasins (68–72)) were produced as a result of the co-culture of *Aspergillus flavipes* and *Streptomyces* sp. which isolated from the same marine sediment. These cytochalasans were reported to be formed by *A. flavipes*, and they were able to have cytotoxic activity against *Streptomyces* sp. at a wide range of concentrations while having no effect on the producer, favoring the producer in competition.⁴⁸ The secondary metabolism of the filamentous model microorganisms *Streptomyces coelicolor* and *Aspergillus niger* is greatly influenced by their co-cultivation. *A. niger* formed the cyclic dipeptide cyclo (Phe–Tyr) (73), cyclo (Phe–Phe) (74), phenylacetic acid (75), furan-2-carboxylic acid (76) and 2-hydroxyphenylacetic acid (77) in reaction to *S. coelicolor*, according to NMR-based metabolomics combined with multivariate data analysis.⁴⁹

3. Elicitation with cell-derived components and alga (Fig. 4)

Many studies have been conducted to induce antibiotic encoding biosynthetic genes in actinomycetes by chemical and biological elicitors, which lead to production of numerous valuable bioactive metabolites based on the whole genome results. Mohammadipanah *et al.* revealed that the supernatant of *Pseudomonas aeruginosa* UTMC 1404 culture could act as stimuli to induce antibacterial synthetic pathways in *Promicromonospora kermanensis* DSM 45485. The eliciting agents in *P. aeruginosa* culture cell filtrate were resistant to detergent, acidic, and basic conditions and had an amphipathic character.⁵⁸

Mixed fermentation of *Nocardia bhagyanarayanae* I-27 and green microalga *Tetradesmus obliquus* AARL G022 yielded essential Ω 3 fatty acids, including eicosapentaenoic acid (97) and α -linolenic acid (98).⁵⁹ Eicosapentaenoic acid granted by the European Commission as Orphan designation for the treatment of familial adenomatous polyposis.

4. Chemical elicitation (Table 4 and Fig. 5)

Antibiotics at sub-inhibitory concentrations (SIC) have been demonstrated to alter gene expression at the transcriptional level, influencing 5–10% of all transcripts. Many phenotypic changes have been seen in the presence of sub-inhibitory antibiotic concentrations, including biofilm formation and enhanced bacterial motility. Antibiotic at SICs are being employed to trigger the production of cryptic natural compounds.⁶⁰ Lincomycin at 1/10 of its MIC significantly boosted the expression of the pathway-specific regulatory gene *act*II-ORF4 in the blue-pigmented antibiotic actinorhodin (ACT) (99) biosynthetic gene cluster, resulting in ACT overproduction in *Streptomyces coelicolor* A3(2).⁶¹

Interestingly, it was found that addition of a SIC (0.1 μ M) of triclosan to the culture media of *S. coelicolor* A3 (2) strain 1147 (wild-type) increased actinorhodin (**99**) synthesis by 5.2-fold. Triclosan had a positive effect on actinorhodin even in the high-producing *S. coelicolor* strain KO-1130 (rpoB), elevating titers by

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Fig. 3 Chemical structures of compounds 45–59 reported upon co-cultivation of actinomycetes with other non-actinomycete bacteria. The configuration of all stereocenters in each compound is shown as given in the literature.

82%. Moreover, addition of a SIC (1 μ M) of triclosan to *Streptomyces albus* industrial strain KO-606 increased salinomycin (**100**) production by 40%, achieving 30.4 g L⁻¹ of salinomycin titer. At subinhibitory concentrations of 0.3 g ml⁻¹ (1/30 of its

MIC), chloramphenicol (ribosome-targeting drug) increased the production of nonribosomal peptide antibiotic (actinomycin D) (**101**) in *S. antibioticus* strain KO-1164 by 1.6-fold CDA by *S. coelicolor* A3(2), presumably *via* increasing the intracellular

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Table 3 Comprehensive list of secondary metabolites reported upon co-cultivation of actinomycetes with fungi

Interaction partners	Induced secondary	Metabolites activity	Reference
Mycobacterium smegmatis	Malformin C (60)	Increase in cytotoxic activity	44
Aspergillus niger			
Streptomyces natalensis	Natamycin (61)	Polyene macrolide polyketide	45
Penicillium chrysogenum	• • • •	antibiotics	
Streptomyces lividans	Bionectriamines A (62)		46
1 9	Bionectriamines B (63)		
<i>Bionectria</i> sp.	Tris(2.4-di- <i>tert</i> -butylphenyl) phosphate		
	(64)		
	6.8-Dihydroxyisocoumarin-3-carboxylic		
	acid (65)		
Streptomyces alhospinus BLe7	Amphotericin B (66)	Antifungal activity	47
Conjochaeta sp. FLe4	milphoteriem B (00)	Induction of red pigmented fungal	17
Somothuotu spi i be i		nhenotype	
Asperaillus flavines	Cytochalasans (67-72)	Cytotoxic effects against Streptomyces	48
Strantomucas sp	Cytoenalasans (07-72)	sp	40
Streptomyces sp.	Cuelo (Dho Tur) (72)	sp.	40
Aspergillus niger	Cyclo (Phe-1yl) (73) $Cyclo (Dhe Dhe) (74)$		49
Aspergilius niger	Cyclo (Pile-Pile) (74)		
	Furen 2 certecurie acid (76)		
	Furan-2-carboxylic acid (76)		
	2-Hydroxyphenylacetic acid (77)		- 0
Streptomyces rochei MB037	Borrelidin (78)	Antibacterial activity	50
Rhinoclaalella similis 35	Borrelidins F (79) , J (80) and K (81)		
	/-Methoxy-2,3-dimethylchromone-4-one		
Strentomyces leeuwenhoekii C34	Luteoride D (83)		51
Aspergillus fumigatus MR2012	Pseurotin G (84)		51
hspergenus junigulus hitt2012	Terezine D (85)		
	11- Ω -methylpseurotin A (86)		
Strentomyces leeuwenhoekii C58	Chavapentin	Inhibit the human lung cancer cell	51
Aspergillus fumigatus MD2012	Dontalonie acid (97)	line A540	51
Aspergilius junigulus MR2012	Dialononsono (82)	IIIIe A349	50
Dhomonoia an EL of	Djatonensone (88)		52
Strontomore niomogenue ASC2D	2,5-Futandinethanoi (89)	Antimicrobial	52
An angillar acistan	Penicisterola C (90)	Antimicropia	53
Aspergillus niger	Haven any whole D (01)	Cytotoxic activities	F 4
Streptomyces sp. CMB-M0423	Heronapyrrole B (91)	Fungistatic	54
Aspergillus sp. CMB-StM0423			
Aspergillus fumigatus	Fumigermin (92)	A reversible germination inhibitor	55
Streptomyces rapamycinicus			_
Trichoderma sp. (307)	(<i>3R</i> , <i>/R</i>)- <i>/</i> -hydroxy-de- <i>O</i> - methyllasiodinlodin (93)	Potent α -glucosidase inhibitory	6
Acinetohacter johnsonii (R2)	(3R)-5-0x0-de-O-Methyllasiodinlodin (04)	activity	
Strentomyces coelicolor $\Delta_3(2)$	Gtri-02 (95)		56
Asneroillus niver NA02			50
Actinomicete WAC 2200	Ibomycin (96)	Anticomptococcus activity	57
Cruntococcus neoformans	10011iyc111 (90)	Anticryptococcus activity	57
Si yprococcus neojormuns			

amino acid pool size.⁶² From GYM agar plates containing various concentrations of streptomycin and paromomycin at doses of 1.5- to 10-fold greater than their respective MICs, 307 spontaneous antibiotic-resistant mutants of *Streptomyces diastatochromogenes* strain 1628 were identified. When streptomycin and paromomycin were used as screening antibiotics, the yields of toyocamycin (**102**), tetramycin A (**103**), tetramycin P (**104**) and tetrin B (**105**) were 244, 680, 583, and 569 mg L⁻¹, respectively, which were 4.1-, 7.8-, 5.1-, and 13- fold higher than the yields of the wild-type strain.⁶³

To uncover cryptic, bioactive metabolites, bioactivity assays are coupled with high-throughput elicitor screening (HiTES). The use of this technique in *Saccharopolyspora cebuensis*, with inhibition of *Escherichia coli* growth as a read-out, resulted in the production of cebulantin (106), a new lanthipeptide, induced by the clinical drugs furosemide and fenofibrate.⁶⁴

HiTES was used successfully to awaken a cryptic gene cluster in *Streptomyces albus* J1074. The cytotoxins etoposide and ivermectin were shown to be powerful inducers, allowing identifying and isolating 14 new small chemical products from the chosen cluster. One of these metabolites is surugamide I (**107**), a strong cathepsin B inhibitor implicated in cancer, while a novel acyl-surugamide A (**108**) had good antifungal activity.⁶⁵ The production of the anthraquinones aloesaponarin II (109) and hypogeamicin B (110) from the actinomycetes *Micromonospora* sp. BBHARD22 and *Nonomuraea* sp. BBHARD23,



Fig. 4 Chemical structures of compounds 60–96 reported upon co-cultivation of actinomycetes with fungi. Cytochalasans: rosellichalasin (67), 19,20-dihydro-aspochalasin D (68), aspochalasin E (69), aspochalasin H (70), aspochalasin M (71), aspochalasin P (72); chemical structures of compounds 97 and 98 reported upon co-cultivation of actinomycetes with alga. The configuration of all stereocenters in each compound is shown as given in the literature.

Table 4 Comprehensive list of secondary metabolites reported upon chemical elicitation

Elicitor	Strain	Secondary metabolites Induced	Mechanism of elicitation	References
Lincomycin	Streptomyces coelicolor A3	Actinorhodin (99) antibiotic	At 1/10 of its MIC, lincomycin significantly boosted the expression of the pathway-specific regulatory gene actII-ORF4 in the blue-pigmented antibiotic actinorhodin (ACT) biosynthetic gene cluster, resulting in ACT overproduction	61
Triclosan	<i>S. coelicolor</i> A3(2) strain 1147 (wild-type)	Actinorhodin (99)		62
Triclosan	Streptomyces albus industrial strain KO-606	Salinomycin (100)		
Chloramphenicol	<i>S. antibioticus</i> strain KO- 1164	Nonribosomal peptide (NRP) antibiotics Actinomycin D (101), calcium- dependent antibiotic (CDA), and piperidamycin)	Chloramphenicol raised amino acid pool sizes 1.5 to 6-fold, boosting CDA and actinomycin production	
Streptomycin and paromomycin	Streptomyces diastatochromogenes 1628	Toyocamycin (102) Tetramycin A (103) Tetramycin P (104) Tetrin B (105)	Ribosome engineering strategies for activating genes for toyocamycin biosynthesis	63
Furosemide fenofibrate	Saccharopolyspora cebuensis	Cebulantin (106) (Inhibitory effect on vibrio strains)	ceb, a class I lanthipeptide BGC	64
Etoposide and ivermectin	Streptomyces albus J1074	Surugamide I (107) inhibit a cysteine protease implicated in cancer Acyl-surugamide A (108) a novel antifungal	Chemogenetic high-throughput screening approach ("HiTES") to discover small molecule elicitors of silent biosynthetic gene clusters	65
Scandium	Micromonospora sp. BBHARD22	Aloesaponarin II (109)		
Streptomycin	Nonomuraea sp. BBHARD23 Microbispora sp. BCCAGE54	Hypogeamicin B (110) Tetarimycin B (111)	Identified through the heterologous production of an environmental-derived type II polyketide synthase (PKS) gene cluster	43
Histone deacetylase inhibitors (hdais)(valproic acid)	Promicromonospora kermanensis DSM 45485		Antibacterial activity	50
Trimethoprim	Burkholderia thailandensis	Acybolins A–I and bactobolin (112- 115)	Antibiotic	66
Ferric ion	Streptomyces sp. FXJ1.172	Antibacterial cyclodepsipeptide, named NC-1 (116)	Lead to the activation of an uncharacterized cyclodepsipeptide, NC-1	67
Rifampicin	Streptomyces somaliensis SCSIO ZH66	Fredericamycin A (117)	Using ribosome engineering and response surface methodology	68
Cl-ARC	Streptomyces ghanaensis ATCC 14672	Oxohygrolidin (118)	Elevate the expression of cryptic biosynthetic genes	69
	Streptomyces hygroscopicus ATCC 53653	9-Methylstreptimidone (119)		
	Streptomyces sp. WAC0256	Cyclotetralactone dynactin Nonactin (23)		

isolated from hypogean cave, increased six-fold under scandium metal exposure conditions. Tetarimycins (**111**) production from *Microbispora* sp. BCCAGE54 was increased thirty-threefold when exposed to streptomycin.⁴³

5. Molecular elicitation

In addition to the cultivation-dependent elicitation techniques previously discussed, a review of metabolic product



Fig. 5 Chemical structures of compounds 99–119 reported upon chemical elicitation. Bactobolins: bactobolin (106), bactobolin A (107), bactobolin B (108), bactobolin C (109).

 Table 5
 Comprehensive list of secondary metabolites reported upon molecular elicitation with transcription factor decoys, overexpression of regulatory genes, promoter replacement strategy, reporter-guided mutant selection and global regulatory genes

Elicitor	Strain	Induced	Mechanism of elicitation	References
Phosphopantheteine transferases (PPtase)	Streptomyces alboniger NRRL B-1832	Puromycin A (120) Puromycin B (121) Puromycin C (122)	Overexpression of PPtases affects secondary metabolism since it regulates primary metabolism, which causes physiological changes and changes in metabolic flux	72
Avenolide	Streptomyces avermitilis	Avermectin (pesticides) (123)		73
TFDs, Rssp8M28I can activate butyrolactol A and Rssp7M24I can activate oxazolepoxidomycin A	<i>Streptomyces</i> sp. F-5635 (ssp8) <i>Streptomyces</i> sp. F-4335 (ssp7)	Butyrolactol A (124) Oxazolepoxidomycin A (125)	The presence of TFDs in the cell can bind to repressors, reducing the interaction between repressors and promoters and resulting in the activation of the silenced genes	74
Overexpression of regulatory gen SARP-type regulator gene	streptomyces sp. SHP22-	Amicetin (126)	Amicetin/plicacetin gene cluster	75
papR2 SigA	7 Corynebacterium glutamicum	Plicacetin (127) β-carotene (128) Bisanhydrobacterioruberin (BABR) (129)	activation induced by PapR2 In the stationary development phase, overexpression of sigA caused a 2-fold increase in the accumulation of the native carotenoid decaprenoxanthin	76
Promoter replacement strategy pCRISPR-Cas9-LigD and pCRISPomyces-2 sgRNA	<i>Streptomyces</i> sp. WAC5374, WAC8241 and 6273	Thiolactomycin (130) Amicetin (126) Phenanthroviridin (131) 5-Chloro-3-formylindole (132)	ligD, an enzyme necessary for NHEJ, and a homology template are both present in the plasmid	4
CRISPR-Cas9 technique	<i>S. roseosporus</i> NRRL 15998	Auroramycin (133)	Utilising a CRISPR-Cas9-based knock-in of KasOp* promoter's silent type I PKS BGC	78
	Streptomyces viridochromogenes	Pentangular type II polyketide (134)	Effective and precise promoter cassette introduction using CRISPR-Cas9 promotes expression of biosynthetic genes and causes the creation of distinctive metabolites that are not seen in the parent strain	83
	S. roseosporus	Macrolactam (135) Photocyclized alteramide A (136) FR-90098 9 (137)		83
Global regulatory genes				-
genes	sireptomyces somaliensis SCSIO ZH66	Violapyrone B (138) Violapyrone A (139) Violapyrone J (140) Violapyrone C (141) Violapyrone H (142)	metabolism and morphological differentiation	79
ΔadpA	Streptomyces ansochromogene	Oviedomycin (143)	adpa, a global regulatory gene, is disrupted to activate a cryptic oviedomycin gene cluster (pks7)	5
adpAa gene inactivation or abrC3 global regulatory gene overexpression	<i>Streptomyces argillaceus</i> ATCC 12956	Antimycin A1a (144) Antimycin A1b (145) Antimycin A2a (146) Antimycin A2b (147) Antimycin A3a (148) Antimycin A3b (149) Antimycin A4a (150)	Regulatory elements that respond to butyrolactone hormone	80

Table 5 (Contd.)

Elicitor	Strain	Secondary metabolites Induced	Mechanism of elicitation	References
		Antimycin A4b (151) Carotenoids (leprotene) (152) 3-Hydroxyleprotene (153) 3,3'-dihydroxyleprotene (154) β-isorenieratene (155) Germicidins B (156) Germicidins C (157) Desferrioxamine B (158)		
Reporter-guided mutant selectio XylE-neo cassette + the promoter of ccaR gene (XylE, a catecholase that dyes colonies brown when catechol is present, was fused to neo, a gene that codes for	n Streptomyces venezuelae ISP5230 and Streptomyces sp. PGA64	Jadomycin B (159) Gaudimycin D (160) Gaudimycin E (161)	Streptomyces spp. reporter-guided mutant selection. The strain is mutagenized using UV light after a double reporter construct called xylE-neo is put under the control of a selected quiet promoter	81
gene, a transcriptional activator of clavulanic acid) Mutagenesis by Tn insertion in conjunction with genetic reporters	Streptomyces albus J1074	Antimycins (144–151) and candicidins (162)	The XNR 3174 gene, which encodes an unidentified transcriptional regulator, was shown to be the site of transposon insertion in the most well-known strain of S. albus,	82
Transposon (Tn) mutagenesis	Streptomyces coelicolor	Tripyrrole antibiotic: Undecylprodigiosin (163) and streptorubin B (RED (164))	ATGSal2P2:TN14. Avenolide-like substance butenolide 4 was discovered to be produced by the mutant strain A hyperactive transposase-based Tn5 transposition system trigger's 724 mutants involving 365 genes, includes 17 genes in the RED biosynthetic gene cluster	84

identification techniques for microorganisms with silent gene clusters discovered through genome mining has been undertaken. Another way to explore the actinomycetes' hidden potential is to mine microbial genomes for silent gene clusters, characterize the cryptic products using gene-knockout experiments, and combine these processes with analytical techniques like MALDI-TOF imaging and HPLC-MS.⁷⁰

5.1. Transcription factor decoys (TFD) (Table 5 and Fig. 6)

Many methods have been devised to fully activate the untapped biosynthetic gene cluster (BGC), either by cloning and expressing the whole biosynthetic gene cluster (BGCs) or by directly modifying regulators in native hosts. Transcription factor decoys are DNA molecules that are created to interfere with gene regulation and may cause both the deactivation of a target BGC that is naturally active as well as the de-repression of a target BGC that is silent.⁷¹ Puromycin A (120), a potent cytotoxic agent, along with two new derivatives (puromycin B and C) (121,122) were identified from *Streptomyces alboniger* NRRL B-1832 by enhancing the phosphopantetheinylation of carrier proteins. To activate the puromycin cryptic/silenced biosynthetic pathway, two broadselective phosphopantetheinyl transferase (PPtase) genes from *Bacillus subtilis* and *S. verticillus* were conjugated into the *S. alboniger* NRRL B-1832 strain.⁷² By altering the expression of a novel TetR-family regulator and its target gene product, it was possible to increase the production of the anthelmintic avermectin in *Streptomyces avermitilis*. In order to promote the production of avermectin (123) and morphological differentiation in *S. avermitilis*, Niu *et al.* 2016 found SAV3619, a TetRfamily transcriptional regulator named AveT, to be an activator.

Transcription Factor Decoys (TFD) activation efficiently activated BGCs from *Streptomyces* sp. F-5635 (ssp8) (a) and *Streptomyces* sp. F-4335 (ssp7), resulting in the isolation of

8



Fig. 6 Chemical structures of compounds 120–125 reported upon molecular elicitation with transcription factor decoys

butyrolactol A (124), a wide antifungal, and a new chemical, oxazolepoxidomycin A (125). $^{74}\,$

5.2. Overexpression of regulatory genes (Table 5 and Fig. 7)

The streptomyces antibiotic regulatory protein (SARP) that regulates PapR2 expression in *Streptomyces lividans* or *Streptomyces* sp. SHP22-7 leads to a significantly improved antibiotic BGC such as: red amicetin (**126**) and plicacetin (**127**) (broadspectrum antibacterial and antiviral disaccharide pyrimidine nucleoside antibiotics), respectively.⁷⁵ β-carotene (**128**) and the non-native bisanhydrobacterioruberin (**129**) production were stimulated by overexpression of the principal sigma factor gene sigA from *Corynebacterium glutamicum*.⁷⁶

5.3. Promoter replacement strategy (Fig. 7)

As a selective and effective method for gene editing, CRISPR/ Cas9 knocks in promoter cassettes to cause the expression of biosynthetic genes. As a result, more strain collections were mined in search of new antibiotics.⁷⁷ (Culp *et al.* 2019). employed CRISPR-Cas9 genome engineering to inactivate the genes producing two of the most prevalent antibiotics, streptothricin and streptomycin, in 11 actinomycete strains. As a result, the modified strain WAC6273 orf17 was used to isolate the uncommon antibiotic amicetin and its new derivatives. Besides, the antibacterial substances thiolactomycin (130), phenanthroviridin (131), and 5-chloro-3-formylindole (132) were discovered utilizing activity-guided purification from two distinct WAC5374 and WAC8241 Δ strI altered strains.⁴

In *Streptomyces roseosporus* NRRL 15998, Lim *et al.* 2018 found auroramycin (133), a strong glycosylated polyene macrolactam antibiotic, by inducing the expression of a 95 kb BGC utilizing a 97 bp kasO* promoter.

5.4. Global regulatory gene (Table 5 and Fig. 8)

Violapyrone B (VLP B) (**138**), a pyrone chemical, was identified when the whiB-like (wbl A50) regulatory gene from *S. somaliensis* SCSIO ZH66, a deep-sea-derived strain, was rendered inactive. Further investigation led to the characterization of the VLP biosynthetic gene cluster and isolation of the VLPs analogues named A (**139**), J (**140**), C (**141**) and H (**142**) as a result of inactivation of vioB.⁷⁹

Global regulatory genes, such as *adpa*, which is the global regulatory gene in several streptomyces species, are activated by genetic manipulation, which results in the activation of cryptic gene clusters. *Adpa* was disrupted in *S. ansochromogenes*, resulting in the development of a mutant whose fermented extract displayed inhibitory zones against Gram-positive bacteria (*S. aureus*, *B. subtilis* and *B. cereus*) in addition to cytotoxic activities which done not occur in the wild type strain which indicate that disruption of *adpa* gene resulted in new metabolites. Chromatographic analysis revealed the presence of the new metabolite, oviedomycin (143) in $\Delta adpa$ fermented extract and absence of nikkomycin traditional produced by the wild type strain *adpa*.⁵



Fig. 7 Chemical structures of compounds reported upon molecular elicitation with overexpression of regulatory genes (126–129) and Promoter Replacement Strategy (130–137).

Becerril et al. adopted four different approaches to activate BGCs in S. argillaceus and isolate encoded compounds; first, they changed culture conditions of S. argillaceus which lead to the production of antimycins (144-151) as a result of the encoded gene cluster 27 being activated. Additionally, they activated the cryptic gene cluster 26 when they expressed it in a heterologous host, which led to the isolation of three new carotenoids (152-155). Furthermore, the discovery of novel secondary metabolites is greatly aided by the global regulatory genes. Herein, the authors inactivated the global regulatory gene adqa resulted in identification of two compounds; germicidins B (156) and C (157) isolated as mixture and decreased production of mithromycin. On the other hand, they overexpressed the global regulatory gene abrc3 in S. argillaceus resulted in the isolation of desferrioxamine (158) (Becerril et al. 2018).

5.5. Reporter-guided mutant selection (Table 5 and Fig. 9)

Reporter-guided mutant selection is one of the most recent approaches (RGMS) for activating silent gene clusters in native producers. It is an efficient and generally applicable technique, in which reporters are employed to aid in the selection of target over-expressing mutants that are then tested for the desired phenotype utilizing reporter gene expression.⁸¹ This approach is predicated on the straightforward idea that the yield of the final metabolite is proportional to the degree of expression of the concerned biosynthetic gene cluster.⁸² Guo *et al.* adopted the RGMS approach in activating two angucycline-type gene clusters that were silent in two strains of *Streptomyces* in standard laboratory conditions. In this report, authors reactivated jadomycin biosynthesis in *Streptomyces venezuelae* ISP5230 lead to isolation of jadomycin B (**159**). In addition, they activated



Fig. 8 Chemical structures of compounds 138–158 reported upon molecular elicitation with global regulatory genes.

a silenced *pga* gene cluster, (accessionno. AY034378) in *Streptomyces* sp. PGA64 resulting in the isolation of two novel anthraquinone aminoglycosides named, gaudimycin D (**160**) and E (**161**). This report validated RGMS as a promising strategy in activating cryptic genes especially after activation of the pga gene cluster which has remained dormant despite more than ten years of continuous efforts using alternative ways.⁸¹

Ahmed *et al.* applied a reporter-guided screening method to activate cryptic polycyclic tetramate macrolactam gene clusters in *Streptomyces albus* J1074. Herein, the low expressed *S. albus* J1074 PTM gene cluster was awakened using a combination of reporter-guided screening and transposon mutagenesis with *gusA's* selection as a reporter resulting in the production of antimycins (**144–151**) and candicidins (**162**).⁸²

Review



Fig. 9 Chemical structures of compounds 159–164 reported upon molecular elicitation with reporter-guided mutant selection.



Fig. 10 The total percentage of isolates from the application of different elicitation methods.

6. Conclusions

Recent advances in the whole-genome sequencing of Actinomycetes has uncovered a wide new field of microbial biology and chemistry. Under ordinary growth conditions, most biosynthetic gene clusters found in genomes were found to be "silent". The scientific world has acknowledged that inactive gene clusters contain a significant source of therapeutic compounds. Several techniques for activating silent BGC have been developed. These methods enable the discovery of new metabolite scaffolds and improve the biosynthesis of minor secondary metabolites. The use of cross-species co-cultures, molecular and chemical elicitation for the biosynthesis of novel natural products have emerged as successful techniques in recent years (Fig. 10). In addition, appropriate dereplication methods, such as molecular networking and metabolomics, are needed to analyze the potential uniqueness of the evoked metabolites and prevent isolating existing compounds. Finally,

novel chemical scaffolds from actinomycetes will be found through structural identification of the new hidden products utilizing LC-MS, MS/MS, and NMR.

Author contributions

A. O. H. gathered a complete survey of all isolated compounds; A. O. H. and M. H. A. H. prepared the manuscript; U. R. A. interpreted and revised the results, and wrote the manuscript; S. S. E., R. M. and U. R. A. scientifically discussed the results and contributed to editing of the manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

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