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REVIEW

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Activating the expression of bacterial cryptic genes by *rpoB* mutations in RNA polymerase or by rare earth elements

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Abstract Since bacteria were found to contain genes encoding enzymes that synthesize a plethora of potential secondary metabolites, interest has grown in the activation of these cryptic pathways. Homologous and heterologous expression of these cryptic secondary metabolite-biosynthetic genes, often “silent” under ordinary laboratory fermentation conditions, may lead to the discovery of novel secondary metabolites. We review current progress on this topic, describing concepts for activating silent genes. We especially focus on genetic manipulation of transcription and translation, as well as the utilization of rare earth elements as a novel method to activate the silent genes. The possible roles of silent genes in bacterial physiology are also discussed.

Keywords Actinomycetes · Cryptic genes · RNA polymerase · *rpoB* mutation · Rare earths · Ribosome engineering

Introduction

Recent advances in DNA sequencing technologies have enabled entire genomes to be sequenced rapidly and inexpensively. Although sequencing of the genomes of *Streptomyces coelicolor* A3(2), *Streptomyces avermitilis*, *Streptomyces griseus*, and *Saccharopolyspora erythraea* showed that each strain contains genes that encode enzymes synthesizing 20 or more potential secondary metabolites [3, 26, 42, 45], only some of these enzymes are produced during fermentation. These cryptic biosynthetic pathways may produce many novel bioactive compounds that can rejuvenate stalled drug discovery pipelines. Thus, as pointed out by Prof. David Hopwood, the number of bacterial secondary metabolites identified to date may be only the tip of the iceberg [21]. Methods to activate these silent biosynthetic pathways are therefore of major interest. The success of this approach depends on finding methods to induce or enhance the expression of cryptic or poorly expressed pathways, yielding material for structural determination and biological testing. This approach can be utilized to solve early stage discovery problems, including (a) inducing some level of expression of cryptic biosynthetic gene clusters (i.e., waking the sleeping genes), and (b) rapidly increasing product yields to obtain sufficient material for chemical and biological evaluation (early stage yield enhancement) [2].

Promising approaches reported to date for the activation of cryptic biosynthetic gene clusters in *Streptomyces* include ribosome engineering; the addition of *N*-acetylglucosamine to the medium or deletion of the *dasR* gene; the constitutive overexpression of a pathway-specific LAL regulatory gene; metabolic remodeling; and cell-to-cell interactions. These new approaches are all applicable to a wide range of actinomycetes and are potentially scalable to high-throughput. In this review, we first outline the “homologous” expression of cryptic genes in actinomycetes. We then discuss the effects of modulating RNA polymerase (RNAP) with *rpoB* mutations, as well as of rare earth elements, to enhance cryptic gene activation. Heterologous expression of cryptic genes, another promising method for mining cryptic secondary metabolite

This article is dedicated to Prof. Sir David Hopwood, who encouraged us mentally and physically throughout his entire research life, in celebration of his 80th birthday.

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pathways, is reviewed by other researchers elsewhere in this issue [61, 64].

Approaches to activate bacterial cryptic genes

Ribosome engineering

The notion of "ribosome engineering" originally came from results showing that a *Streptomyces lividans* strain with an altered ribosomal S12 protein that confers streptomycin resistance produced abundant quantities of the blue-pigmented antibiotic actinorhodin, although *S. lividans* normally does not produce antibiotics due to the dormancy of the antibiotic biosynthesis genes [50]. Furthermore, the bacterial alarmone ppGpp, produced on ribosomes in response to nutrient starvation, was found to bind to RNAP [1], eventually initiating the production of antibiotics [4, 38]. These results suggested that modifying RNAP, for example, by introducing a rifampicin resistance mutation, may mimic the ppGpp-bound form of RNAP with an enhanced affinity to promoter region of the secondary metabolite synthesis-regulatory genes, thus activating the expression of biosynthetic gene clusters [33, 60]. We hypothesized that bacterial gene expression may be increased dramatically by altering transcription and translation pathways. To address this hypothesis, we developed a method, termed ribosome engineering, to activate or enhance the production of secondary metabolites by targeting ribosomal protein S12, as well as other ribosomal proteins and translation factors, or RNAP. Ribosome engineering is applicable to both silent gene activation and strain improvement methods of identifying novel secondary metabolites [22, 25, 29, 62], as well as to the enhancement of enzyme production [31, 32] and tolerance to organic chemicals [24]. Combinations of these drug-resistance mutations can further enhance bacterial productivity [58]. The fundamental mechanism by which ribosome engineering affects antibiotic production has been summarized in earlier reviews [23, 38, 41] as has many successful examples [39] and the outline of this technology [2, 8, 44].

dasR—*N*-acetylglucosamine system

DasR is a global regulator of antibiotic biosynthesis, linking nutrient stress to antibiotic production by *Streptomyces*. A high concentration of *N*-acetylglucosamine (GlcNAc), perhaps mimicking its accumulation after autolytic degradation of the vegetative mycelium, may be a major checkpoint for the onset of secondary metabolism [48]. The response is transmitted to antibiotic pathway-specific activators through the pleiotropic transcriptional repressor DasR. GlcNAc also stimulated antibiotic production by other *Streptomyces* spp., including *S. clavuligerus*, *S. collonus*, *S. griseus*, *S. hygroscopicus*, and *S. venezuelae* [48]. Interestingly, the *dasR* mutant BAP29 was found to awaken cryptic gene clusters encoding a hypothetical antibiotic synthesized by a type I modular polyketide synthase, showing that this method is applicable to the activation of silent gene clusters [64]. For further details, we refer to the review by Gilles van Wezel and colleagues elsewhere in this issue.

LAL regulatory system

Constitutive overexpression of a putative pathway-specific LAL (large ATP-binding LuxR-type) regulator was shown to successfully induce the expression of the silent type I modular PKS gene cluster in *Streptomyces ambofaciens* 23877, enabling the identification of a unique structural class of polyketides with promising antitumor activity [34]. The constitutive expression of a regulatory gene within this cluster, encoding a protein similar to the LAL family of proteins, triggered the expression of the biosynthetic genes, suggesting that constitutive expression of such pathway-specific activators represents a powerful approach for the discovery of novel bioactive natural products [34].

The metabolism-remodeling approach

Although activation of biosynthetic genes at the transcriptional level is of prime importance in exploiting useful metabolites, perturbation of biosynthesis by modulating, for example, the supply of precursors may also be a promising approach to enhancing the cell's capability to produce secondary metabolites. The yields of secondary metabolites may be enhanced by small molecules that modulate fatty acid biosynthesis [10, 61]. For example, screening of a large number (>30,000) of small molecules, seeking candidates that might "remodel" the yields of actinorhodin in *S. coelicolor*, identified 19 compounds that caused elevated or precocious production of actinorhodin [11]. Since fatty acid and polyketide synthesis share the precursors acetyl-CoA and malonyl-CoA, partial inhibition of fatty acid synthesis may recruit these acyl-CoAs preferentially to polyketide biosynthesis. Thus, the relationship between polyketide and fatty acid biosynthesis is very intimate. Vegetable oils are often used in industrial antibiotic fermentation processes as preferable carbon sources. Use of these slowly metabolized carbon sources may avoid carbon catabolite repression and may increase yields of antibiotics. Fatty acids derived from vegetable oils are metabolized via the β-oxidation pathway to generate acetyl-CoA. The generation of abundant quantities of acetyl CoA from vegetable oils may perturb fatty acid metabolism, redirecting common substrates to secondary metabolic pathways. Theoretically, this approach may

be enhanced by combination with transcription-associated approaches [40]. For further reviews, we refer to the review by Justin Nodwell and colleagues elsewhere in this issue.

Cell-to-cell interactions

Cell co-culture is effective in inducing the production of cryptic metabolites. Although some co-culture methods have been reported, these methods are often specific to two specific bacterial strains. These limitations, however, may be overcome by a novel fermentation method, the combined-culture method, involving the co-culture of two bacterial strains [46]. For example, the production of red pigment by *S. lividans* is induced by co-culturing these bacteria with *Tsukamurella pulmonis*, a mycolic acid-containing bacterium. Importantly, co-culture with *T. pulmonis* of *Streptomyces* strains isolated from soil altered the synthesis of natural products in 88 % of these bacterial strains, with the production of new secondary metabolites detected in 37 % and increased metabolite production in 55 %. Co-culture with *Rhodococcus erythropolis* and *Corynebacterium glutamicum*, two species of mycolic acid-containing bacteria, altered natural product biosynthesis in 87 and 90 % of the *Streptomyces* strains, respectively, with the production of new secondary metabolites detected in 32 and 24 % of strains, respectively. Thus, mycolic acid-containing bacteria can influence the biosynthesis of cryptic natural products in *Streptomyces* spp. Co-culture of *T. pulmonis* with *Streptomyces endus* led to the identification of a novel antibiotic, alchivemycin A. In contrast, the addition of mycolic acid to the medium of pure *S. lividans* cultures had no effect on antibiotic production, suggesting that mycolic acid localized to the outer cell layer of the inducer bacterium affected secondary metabolism in *Streptomyces*, with this activity resulting from direct cell-to-cell interaction of the two bacterial strains [46]. Although the functional mechanism underlying this method is enigmatic, the approach is easily scalable for the production of cryptic antibiotics, as it only involves the addition of a mycolic acid-containing bacterium to a pure culture of an actinomycete.

Activation of cryptic genes by *rpoB* mutations

Ribosome engineering is effective for the activation of silent genes. Certain mutations in the *rpoB* (encoding RNAP β-subunit) and *rpsL* (encoding ribosomal protein S12) genes, which confer resistance to the antibiotics rifampicin and streptomycin, respectively, can activate silent or weakly expressed genes of actinomycetes or *Bacillus subtilis*, leading to the discovery of novel antibacterial agents [22, 29]. We also found that *rsmG* mutations, which are responsible for a low level of resistance to streptomycin [36, 37, 43], cannot only activate streptomycin production but the expression of other secondary metabolite-biosynthetic genes in *S. griseus* [54, 55]. For example, among the 1,068 actinomycetes isolated from soil, some of the *Streptomyces* and most of the non-*Streptomyces* isolates were found to be nonproducers of antibiotics [22]. Of these, 43 and 6 %, respectively, were able to synthesize antibacterials against *Staphylococcus aureus* after a selection step that generated spontaneous *rpsL* or *rpoB* mutations [22]. Assessment of *Streptomyces mauvecolor* 631689, a strain with no antibacterial activity in any medium tested, demonstrated that two *rpoB* mutants (H437D or H437L), a double mutant of *rpoB* (H437L) and *rpsL* (K88R), and a gentamicin-resistant (GenR) mutant produced a new family of antibiotics, the piperidamycins. The activation of silent genes by the *rpoB* H437D or H437L mutations was attributed, at least in part, to the increased affinity of mutant RNAP for silent gene promoters [22]. Species of *Bacillus* produce a variety of commercially important metabolites and extracellular enzymes. The introduction of the *rpoB* mutation S487L (corresponding to S433L of *S. coelicolor*) into a *B. subtilis* strain also yielded cells that overproduced an aminosugar antibiotic, 3,3′-neotrehalosadiamine (NTD), the production of which is dormant in the wild-type strain [29]. Drug-resistance mutations in *Nocardiopsis* sp., such as those conferring resistance to streptomycin or rifampicin, result in broad changes in metabolic phenotype as well as secondary metabolism of these organisms [13].

Broad applicability of the *rpoB* mutation method

The broad applicability of the *rpoB* mutation method to the expression of cryptic secondary metabolite-biosynthetic gene clusters was demonstrated recently [56]. A total of 18 genes belonging to 18 secondary metabolite-biosynthetic gene clusters of *S. griseus* (Table 1) were subjected to transcriptional analysis by real-time quantitative PCR (real-time qPCR), with the wild-type and *rpoB* mutant strains compared (Fig. 1a). RNAs were extracted from cells grown to late growth phase and the maximum levels of expression detected in each culture were compared (Fig. 1b). Of the *rpoB* mutants examined, strain KO-1172 with an H437Y mutation showed marked activation of cryptic genes at the transcriptional level. This was especially pronounced for SGR3267, SGR4413, and SGR5295, which were activated 50 to 70-fold. Although the H437Y mutation was effective in both 2 × GYM and R4 medium, the H437R mutation was effective only in 2 × GYM medium. Thus, the activation of silent gene clusters by *rpoB* mutations was medium-dependent, with each *rpoB* mutation having differential effects on the activation of each silent gene cluster. Conceivably, the expression of each silent gene cluster is controlled by multiple factors, all of which may be affected,

Table 1 Cryptic secondary metabolite-biosynthetic genes of *S. griseus* IFO13189 analyzed

Gene	Product	Secondary metabolite-biosynthetic gene cluster
SGR281	Hypothetical protein	PKS-NRPS hybrid (SGR278–SGR283)
SGR443	Putative ABC transporter ATPase and permease component	NRPS for siderophore (SGR443–SGR455)
SGR593	Hypothetical protein	NRPS (SGR574–SGR593)
SGR604	Putative enediyne biosynthesis protein	Enediyne PKS (SGR604–SGR611)
SGR811	Putative oxidoreductase	PKS-NRPS hybrid (SGR810–SGR815)
SGR896	Putative O-methyltransferase	NRPS (SGR895–SGR901)
SGR962	Putative squalene–hopene cyclase	Hopanoid (SGR962–SGR966)
SGR2079	Putative terpene cyclase	Terpene (SGR2079)
SGR2488	Putative dehydrogenase	Type I PKS, NRPS (SGR2482–SGR2489)
SGR2594	Putative integral membrane ion antiporter	NRPS (SGR2586–SGR2598)
SGR3267	Putative cytochrome P450	Type II PKS, NRPS (SGR3239–SGR3288)
SGR4413	Putative lantibiotic biosynthesis protein	Lantibiotic (SGR4408–SGR4421)
SGR5295	5-Aminolevulinate synthase	Unknown (SGR5285–SGR5295)
SGR6072	Putative ketosteroid isomerase	Type I PKS (SGR6071–SGR6083)
SGR6178	Putative thioesterase	Type I PKS (SGR6177–SGR6183)
SGR6367	Putative oxidoreductase	Type I PKS (SGR6360–SGR6387)
SGR6717	Putative ABC transporter ATPase and permease component	NRPS for siderophore (SGR6709–SGR6717)
SGR6780	Putative malonyl-CoA:ACP transacylase	Type I PKS, PKS-NRPS hybrid (SGR6776–SGR6786)

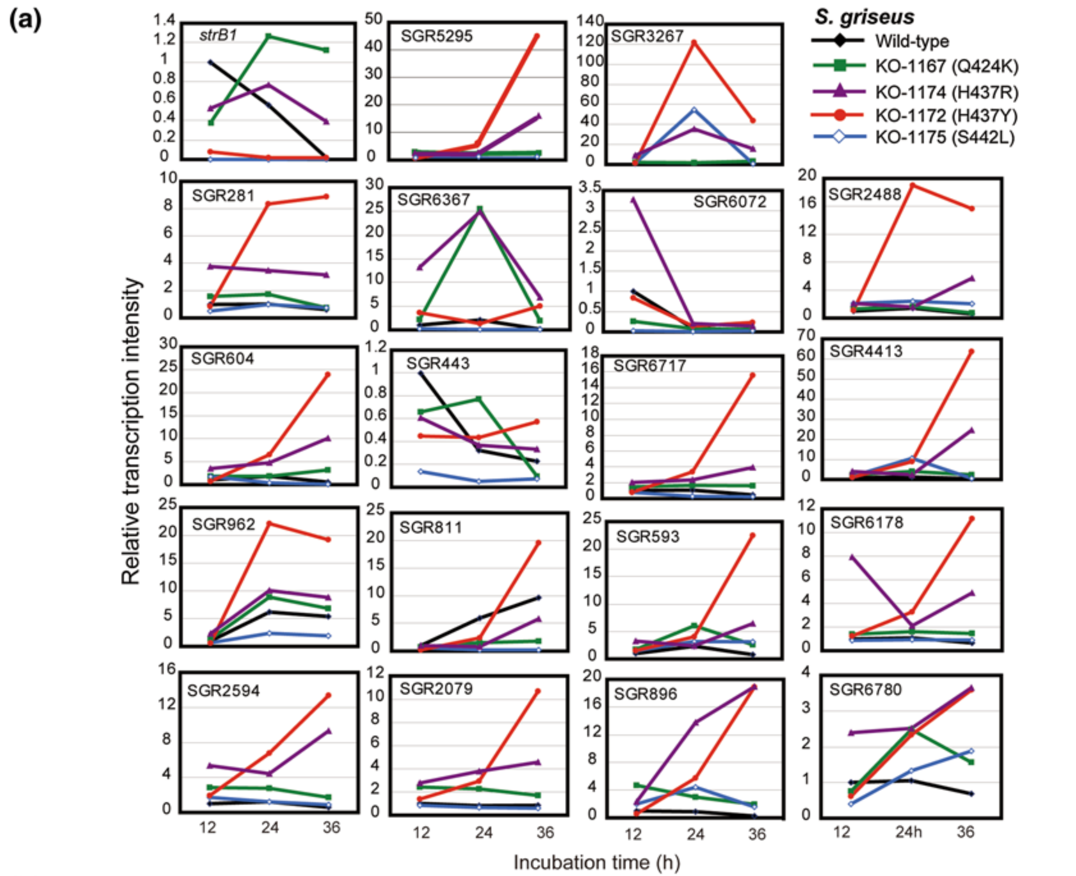
From Ohnishi et al. [42]

both qualitatively and quantitatively, under various culture conditions. These findings suggest that strains containing different *rpoB* mutations (e.g., H437Y, H437R) should be grown in different media to access the full spectrum of their activities in silent gene activation. These results, in turn, may show that modulation of downregulated genes, as represented by the pathway-specific positive regulatory genes *strR* [47] and *actII*-ORF4 [18] in streptomycin and actinorhodin production, respectively, is more effective in inducing antibiotic overproduction by *rpoB* mutants.

S. erythraea is a widely studied model system for antibiotic production. BldD, a key developmental regulator in actinomycetes [15], regulates the synthesis of erythromycin [9]. The *rpoB* H437R mutation was found to markedly (6.5-fold) enhance *bldD* expression, leading to the upregulation of a biosynthetic gene (*eryCII*) and erythromycin overproduction. Increased erythromycin production may also be due, at least in part, to the effects of *rpoB* H437R on the expression of genes encoding key enzymes involved in carbon and nitrogen metabolism, activating erythromycin feeder pathways [6]. The observed upregulation of cryptic genes by *rpoB* mutations (Fig. 1b) may be due, at least in part, to the increased affinity of mutant RNAP for the silent gene promoters. In contrast, the down regulation of expression of several cryptic genes may result from the reduced affinity of mutant RNAP for the promoters.

In *S. coelicolor* and *S. erythraea*, a total of 15 genes belonging to 15 cryptic secondary metabolite-biosynthetic genes clusters were subjected to transcription analysis. As expected, the H437Y and H437R mutations, which were effective in enhancing erythromycin production, were widely effective in activating the cryptic genes of *S. erythraea*, with six of the 15 genes examined showing a $\geq$ 3-fold increase in transcription. Similarly, the S433L and H437Y mutations, which were effective in enhancing actinorhodin production, effectively activated the cryptic genes of *S. coelicolor* [56]. Amino acid alterations at the H437 position (corresponding to positions H406, H482, and H526 in *Thermus thermophilus*, *B. subtilis* and *Escherichia coli*, respectively) were often effective in activating cryptic pathways. The mutations at position H437 have been shown to circumvent the detrimental effects of *relA* and *afsB* mutations (in *S. coelicolor*) and *relC* mutation (in *S. lividans*) on actinorhodin production, perhaps

Fig. 1 Transcriptional and metabolite analysis of the genes included in the secondary metabolite-biosynthetic gene clusters in *rpoB* mutant strains of *S. griseus*. **a** Profile of changes in expression of secondary metabolite-biosynthetic genes of *S. griseus rpoB* mutants grown in 2 × GYM medium. Total RNA was extracted from cells grown at 25 °C for 12, 24, and 36 h, followed by real-time qPCR analysis. Levels of expression were normalized relative to those in the wild-type strain IFO13189 at 12 h (=1). **b** Comparison of the maximum expression levels in each culture. Levels of expression were normalized as in (**a**). **c** 2D-UPLC/MS analysis of the metabolite profile of *rpoB* mutants. *S. erythraea* and *S. griseus* strains were grown for 2 and 4 days, respectively, in 2 × GYM medium, and *S. coelicolor* was grown for 4 days in GYM medium. The results are shown as 2D plots [positive ions (*m/z*) vs. retention time (min)]. The spots detected in the wild type are marked by *black circles*, while those not detected in the wild type are marked by *red circles*. Data are from Tanaka et al. [56] (color figure online) ▶


(a)
S. griseus
Wild-type
KO-1167 (Q424K)
KO-1174 (H437R)
KO-1172 (H437Y)
KO-1175 (S442L)
strB1
SGR5295
SGR3267
SGR281
SGR6367
SGR6072
SGR2488
SGR604
SGR443
SGR6717
SGR4413
SGR962
SGR811
SGR593
SGR6178
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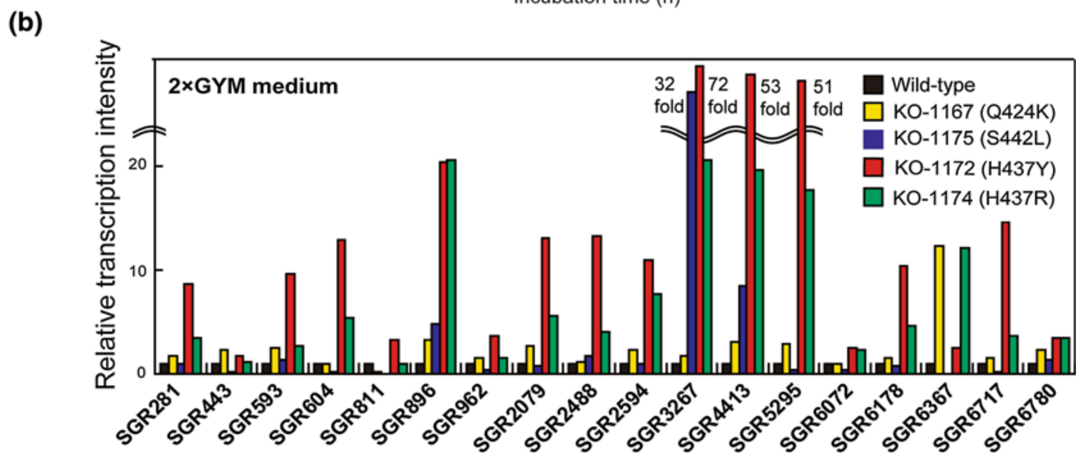
SGR2079
SGR896
SGR6780
Relative transcription intensity
Incubation time (h)


(b)
2×GYM medium
32 fold
72 fold
53 fold
51 fold
Wild-type
KO-1167 (Q424K)
KO-1175 (S442L)
KO-1172 (H437Y)
KO-1174 (H437R)
Relative transcription intensity
SGR281
SGR443
SGR593
SGR604
SGR811
SGR896
SGR962
SGR2079
SGR2488
SGR2594
SGR3267
SGR4413
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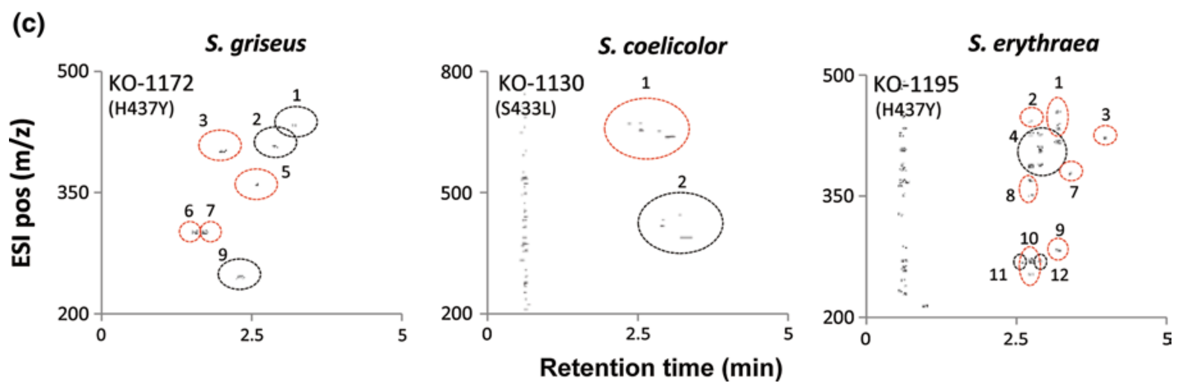
SGR6072
SGR6178
SGR6367
SGR6717
SGR6780


(c)
S. griseus
S. coelicolor
S. erythraea
KO-1172
(H437Y)
KO-1130
(S433L)
KO-1195
(H437Y)
ESI pos (m/z)
Retention time (min)

by mimicking the ppGpp-bound form of RNAP [33, 52, 60]. These findings indicate that the bacterial alarmone ppGpp likely participates significantly in the activation of cryptic gene clusters as well as in the activation of highly expressed, well known, secondary metabolite gene clusters. The precocious overexpression of cryptic gene clusters and cell lysis observed during the late growth phase of *S. griseus* containing the *rpoB* H437Y mutation in certain media may be explained by its unique spectrum of effects in modulating the transcription of each gene. Indeed, the *S. erythraea rpoB* S444F mutation, corresponding to S442F in this study, markedly altered the transcriptional profile of this organism [6].

The *rpoB* mutants actually produce newly synthesized metabolites

In addition to markedly activating cryptic genes, *rpoB* mutations enabled these cells to produce a number of metabolites not detected in the wild-type strain, as demonstrated by 2D-UPLC/MS analyses (Fig. 1c). This was especially pronounced in *S. griseus* KO-1172 (H437Y), *S. coelicolor* KO-1130 (S443L), and *S. erythraea* KO-1195 (H437Y), as indicated by red circles in Fig. 1c. The *rpoB* mutation resulting in the highest yield of metabolite varied by metabolite, although the H437Y mutation was frequently optimal [56]. These results demonstrated that introducing *rpoB* mutations not only activated the expression of cryptic genes at the transcriptional level but may be effective in identifying novel secondary metabolites.

Utilization of "natural" mutant-type RNAP genes

The recent observation that several actinomycetes possess two *rpoB* genes [57] has suggested a new strategy of activating silent gene expression in bacteria. Two *rpoB* paralogs, *rpoB(S)* (rifampicin-sensitive wild-type *rpoB*) and *rpoB(R)* (rifampicin-resistant mutant-type *rpoB*), provide *Nonomuraea* sp. strain 39727 with two functionally distinct and developmentally regulated RNAPs. The product of *rpoB(R),* the expression of which increases after transition to stationary phase, is characterized by five amino acid substitutions (H426 N, S431 N, F445 M, S474Y, and M581D) located within or close to the rifampicin resistance cluster. The expression of *rpoB(R)* was found to markedly activate antibiotic biosynthesis, with the *rpoB(R)*-specific H426N mutation essential in activating secondary metabolism [52]. Other *rif* cluster-associated *rpoB(R)*-specific missense mutations likely interact functionally with the H426N mutation, leading to the marked effect of *rpoB(R)*. Mutant-type, or duplicated, *rpoB* often exists in nature, with *rpoB* gene polymorphisms detected in 5 of 75 inherently rifampicin-resistant actinomycetes isolated from nature, although these polymorphisms were preferentially distributed in the so-called rare actinomycetes, not in *Streptomyces* spp. Notably, all but one of the rifampicin-resistant rare actinomycete isolates identified to date were able to produce antibiotics [52]. Moreover, the results working with *Nonomuraea terrinata* strains with single *rpoB* [*rpoB*(R)] or duplicated *rpoB* [*rpoB*(S) + *rpoB*(R)] demonstrated that the *N. terrinata* strain with duplicated *rpoB* shows much greater capability than the single *rpoB* strain for growth (representing primary metabolism) and sporulation and antibiotic production (representing the developmental strategy), especially under stressful conditions, thus suggesting the physiological significance of *rpoB* duplication [52]. Therefore, when constructing the host organism for heterologous gene expression, coexistence of mutant-type and wild-type *rpoB* genes may be more preferable to existence of mutant-type *rpoB* gene alone because the disadvantage in growth rate caused by *rpoB* mutation could be circumvented with the presence of the wild-type *rpoB* gene. From a practical viewpoint, these findings suggest the intriguing possibility of using *rpoB(R)*-based technology to improve strains and to search for novel bioactive molecules by activating silent genes. This technology should have greater potential than the simple *rif* selection method currently used to improve the production of secondary metabolites, as the introduction of *rpoB(R)* enhanced antibiotic production eight-fold compared with the H426Y mutation [52]. It is also of interest to assess whether various *rpoB(R)* forms found in nature are more capable of activating silent bacterial genes than *Nonomuraea rpoB(R)*. Thus, understanding the status of natural *rpoB(R)* and utilizing it for cryptic gene activation or antibiotic overproduction may provide new horizons for medical and industrial microbiology.

SGR3267 and SGR5295 cannot be activated under general stress conditions

It is widely accepted that bacterial secondary metabolism and morphological differentiation is usually triggered when cells encounter adverse environmental conditions, including nutrient limitations or the presence of stress stimuli [4, 14, 38]. As the *S. griseus* cryptic genes SGR3267 and SGR 5295 were markedly activated by introducing certain *rpoB* mutations (see above), we were interested in determining whether these cryptic genes can be activated under certain stressful conditions. We therefore analyzed SGR3267 and SGR5295 transcription in cells subjected to 32 different stress conditions, followed by culture for an additional 3 h (Fig. 2a). The stress stimuli included pH changes, temperature shifts, and addition of chemicals such as heavy metals, antibiotics, and flavonoids. As heavy metals and

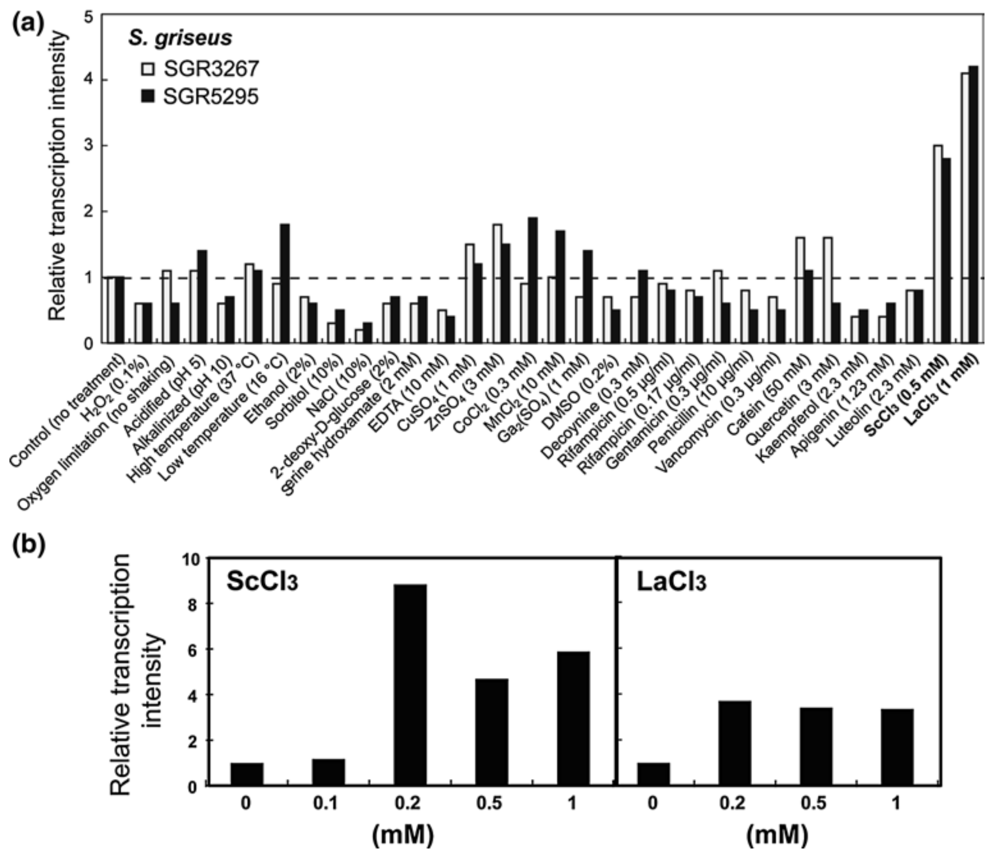


Fig. 2 Transcriptional analysis of *S. griseus* cryptic genes SGR 3267 and SGR5295 under various stressful conditions. **a** *S. griseus* IFO13189 (wild-type strain) was grown in 2 × GYM medium for 12 h, subjected to stress conditions, and incubated for an additional 3 h. RNAs were extracted and analyzed by real-time qPCR. Levels of expression were normalized relative to those of untreated cells (=1). **b** Effects of $ScCl_3$ and $LaCl_3$ concentrations on the expression of cryptic gene SGR3267. Experimental conditions were identical to those in (**a**). The mean values of two samples are shown

antibiotics are potent growth inhibitors, these chemicals were added at sublethal concentrations, i.e., one-third of their minimum inhibitory concentrations (MIC). The SGR3267 and SGR5295 genes were activated by rare earth elements, but not by any of the other stress conditions. Treatment with rifampicin was also ineffective. Although certain flavonoids possess antibacterial activity and induce the expression of certain bacterial genes [20], none of the flavonoids tested had significant effects on the expression of *S. griseus* cryptic genes (Fig. 2a). Similarly, expression of the SGR3267 and SGR5295 genes was not significantly affected by heavy metals, such as Cu, Zn, Co, Mn, and Ga. Thus, the effect of rare earth elements on activation should be highlighted (see below). These results indicate that the SGR3267 and SGR5295 genes are "silent" under ordinary laboratory fermentation conditions and, in turn, emphasize the efficacy of certain *rpoB* mutations that resulted in 50 to 70-fold activation.

Applicability of rare earth elements to biotechnology

The rare earth elements (REEs) consist of 17 elements, including scandium (Sc), yttrium (Y), and the lanthanides (i.e., the 15 elements from lanthanum [La] to lutetium [Lu] in the periodic table). Despite the importance of REEs in the chemical industry, little is known about their biological effects in living cells. These elements, however, have recently been shown involved in the overproduction of antibiotics and in the activation of silent genes in bacteria and in plant's physiology.

Applicability to microorganisms

All the REEs were effective in enhancing actinorhodin production, with the effect of Sc being the most pronounced. In contrast, the chloride salts of manganese (Mn), iron (Fe), nickel (Ni), copper (Cu) and zinc (Zn) were ineffective (Fig. 3a). Low concentrations (10–100 μM) of Sc added to cultures of *S. coelicolor*, *S. griseus* and *Streptomyces antibioticus* were found to enhance antibiotic production 2 to 25-fold [30]. Sc enhanced the transcription of pathway-specific positive regulatory genes, as demonstrated by marked up-regulation of *actII*-ORF4 in *S. coelicolor*. Moreover, the addition to the medium of low concentrations of Sc or La activated the expression of nine genes, 2.5 to 12-fold, belonging to nine cryptic secondary metabolite-biosynthetic gene clusters [53]. HPLC analysis of ethyl acetate-extractable metabolites indicated that several compounds were detected only in REE-treated cultures (Fig. 3b). Strikingly, addition to the medium of sublethal concentrations (one-third of the MIC) of $ScCl_3$ or $LaCl_3$ substantially

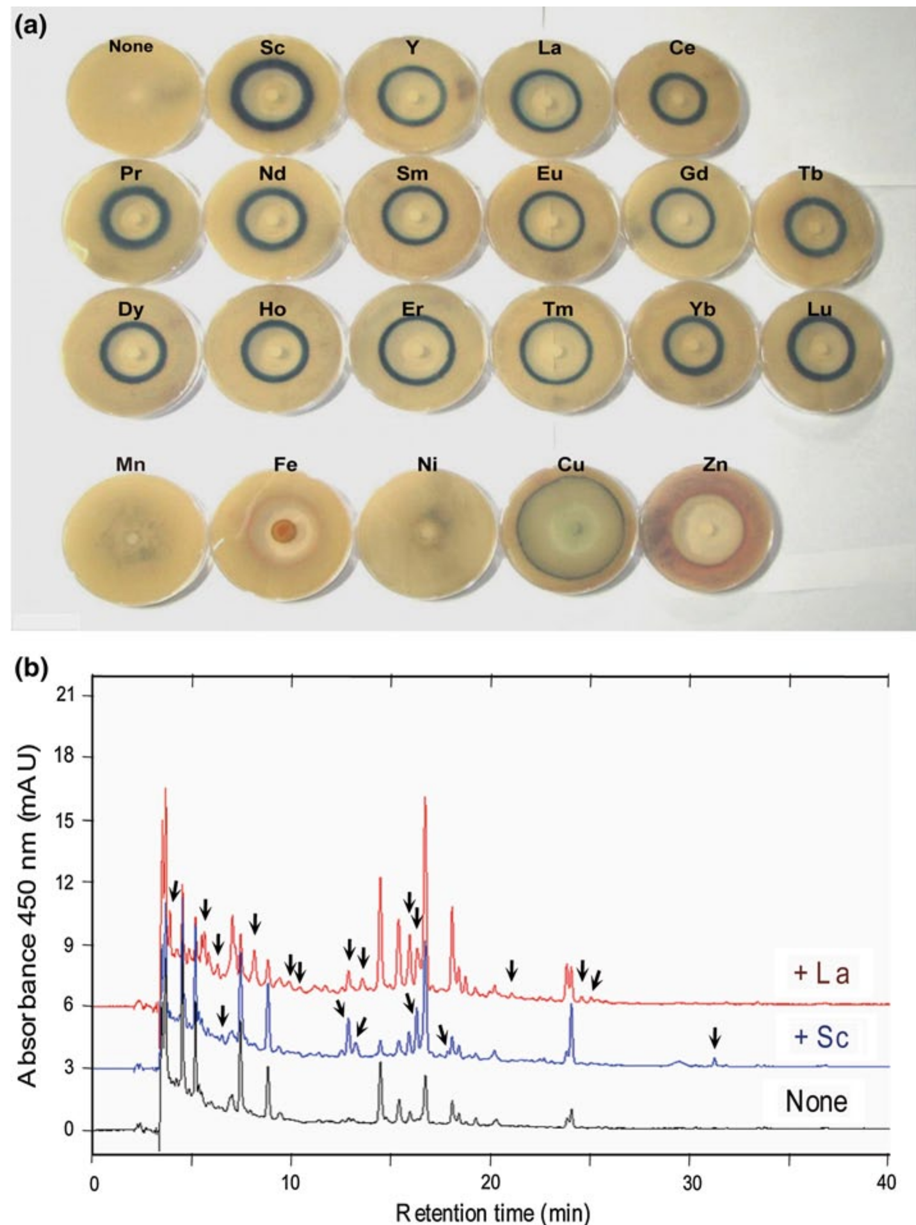


Fig. 3 REE activation of secondary metabolite production. **a** Effects of exogenously added REEs on actinorhodin production. *S. coelicolor* wild-type strain 1,147 was spread on GYM agar, and a paper disk containing 4 mg of each rare earth or heavy metal (all chloride salts) was placed in the center of the plate. The plates were incubated at 30 °C for 4 days. *Blue* represents the antibiotic actinorhodin, whereas the clear zone at the center represents growth inhibition. **b** Comparative metabolic profiling of the culture extracts. *S. coelicolor* 1,147 was grown on GYM agar in the presence or absence of $ScCl_3$ (0.2 mM) or $LaCl_3$ (1.9 mM) for 5 days. Culture were extracted with EtOAc and analyzed by HPLC. The *arrows* indicate the peaks scarcely detected in the absence of REEs. Data are from Tanaka et al. [53] (color figure online)

enhanced the transcription of silent genes, SGR3267 and SGR5295, of *S. griseus* (Fig. 2a), distinguishing the REEs as stress stimuli. Expression of the SGR3267 gene was optimally induced by 0.2 mM $ScCl_3$ or $LaCl_3$, with the former showing ninefold activation (Fig. 2b). This approach, due to its feasibility, should facilitate the discovery of new biologically active compounds. The ability of REEs, especially Sc, to enhance enzyme production and secondary metabolism was also observed in *B. subtilis*. The addition of Sc to the growth medium stimulated the transcription of genes encoding both α-amylase and the antibiotic, bacilysin [27]. An important advantage of using REEs is that this method does not require any gene engineering technology or genomic information on the strains examined. It is, however, notable that effective concentrations of REEs to activate antibiotic production differ in the order of magnitude among REEs and are in a rather narrow range [53].

Microorganisms have a high REE biosorption capacity [35, 51], and siderophores may play a role in the accumulation of REEs, as shown by siderophore-forming *Arthrobacter luteolus* isolated from REE-rich environments [7]. REE ions are adsorbed efficiently onto functional groups present in bacterial cell walls, such as the phosphate and carboxyl groups of lipoteichoic acid [35, 51]. Since REEs are weakly antimicrobial, it was possible to develop Sc-resistant mutants on plates containing Sc. As expected, many of the Sc-resistant mutants of *S. coelicolor* and *B. subtilis* were overproducers of actinorhodin (Ochi K, unpublished

results) and α-amylase [28], respectively. In *B. subtilis*, a mutation in the *uppS* gene, which encodes the enzyme undecaprenyl pyrophosphate synthase (involved in cell wall synthesis), was responsible for the Sc resistance phenotype and α-amylase overproduction. This *uppS86* mutation, however, did not affect the levels of *amyE* expression, suggesting that this mutation exerted its effects at the post-transcriptional or physiological level [28]. Thus, the mechanisms by which the *uppS86* mutation conferring Sc resistance and the addition of Sc stimulated α-amylase production differed.

Applicability to plants and animals

REEs have also shown interesting biological effects on plants and have been utilized in agricultural fertilizers for more than 30 years in China. REEs can exert positive or negative physiological effects on plants, depending on the dosage and other conditions. In particular, there is increasing interest in applying REEs to medicinal plants ([63] and references therein). Appropriate concentrations of REEs can promote seed germination and root development, increase plant biomass, and improve the quality of fruiting bodies [5, 12], whereas too high concentrations can inhibit plant growth and even cause death. Appropriate concentrations of REEs can increase the chlorophyll content of leaves and promote plant growth. Moreover, REEs were shown to increase the frequency of *Agrobacterium tumefaciens*-mediated plant transformation [5] and may regulate the absorption of other mineral elements into plants [63]. The strong positive impact of REEs on plant growth may be due, however, to accelerated nitrogen metabolism [5]. However, results of field trials and laboratory studies are still unclear, with results depending on the plant species, physiological stage and type of treatment. In particular, REEs promote the biosynthesis of secondary metabolites, such as flavonoids, isoflavones and benzyl ethanol glycosides, in medicinal tissue-cultured plants (and even in cultured plants) by activating the transcription of essential biosynthetic genes [63], results parallel to the effects of REEs in bacteria. Although REEs may improve animal growth [19], high concentrations of REEs show in vitro cytotoxicity (e.g., anti-carcinogenic properties) in mammalian cells at high concentrations [16]. Detailed investigations are needed to determine the effects of REEs on the environment, plants, and humans.

Why silent genes?

Apart from the technology, it is important to determine why cryptic genes are not expressed under laboratory fermentation conditions. It is unclear whether silent genes are expressed under special, as yet-unknown environmental conditions, or if the cryptic secondary metabolites play an intrinsic biological role(s) in producing organisms under these special environmental conditions. Determination of any special environmental conditions, as well as understanding the mechanism(s) underlying the silencing of cryptic genes, would help to fully utilize microbial gene clusters for secondary metabolism. Studies showing the effects of REEs on cryptic gene activation may be a clue to address these questions. Since REEs are distributed ubiquitously throughout the world, microorganisms may have acquired the ability to respond to low levels of these elements as an "abiotic" stress over their long evolutionary history, possibly as a means of adapting to prevailing conditions. REEs have much more potent growth inhibitory effect than the related heavy metals, such as Cu, Zn, and Mn. The effects of low concentrations of Sc on antibiotic production indicate that Sc functions in situ as a factor that induces or stimulates the production of secondary metabolites, including pigments, mycotoxins, phytotoxins, and antibiotics.

Another clue may be provided by cryptic gene activation through cell-to-cell interactions [46]. Mycolic acid-containing actinomycetes, such as *Rhodococcus*, *Corynebacterium*, *Mycobacterium*, and *Tsukamurella*, are widely distributed in soil habitats. It is possible that the presence of mycolic acid-containing actinomycetes can have deleterious effects on the growth of neighboring actinomycetes. Thus, the latter may produce cryptic gene-derived secondary metabolites to prevent the proliferation of mycolic acid-containing actinomycetes. Alternatively, the population of mycolic acid-containing actinomycetes may be a marker of certain, as yet undetermined, types of "biotic" stress. Since some *rpoB* mutations were unable to activate several cryptic genes in *S. griseus*, it is of interest to compare the spectrum and level of activation of cryptic genes due to cell-to-cell interactions, *rpoB* mutations and REE addition.

The presence of "natural" mutant type RNAPs, either as polymorphisms or non-polymorphisms, may also be used to resolve questions about cryptic genes [52]. Although <0.1 % of actinomycetes in the soil have a natural mutant-type RNAP gene, their cryptic genes are apparently expressed during late growth phase. Therefore, it is likely that, in the absence of the natural mutant-type RNAP gene, any cryptic genes in actinomycetes would be expressed under as yet-unknown, specified, environmental conditions and that activation of these secondary metabolite-biosynthetic pathways may enhance their survival advantage. For example, certain cryptic genes (SGR3267, SGR4413, and SGR5295) of *S. griseus* are expressed intensively under special environmental conditions, inasmuch as they are activated 50 to 70-fold by certain *rpoB* mutations (see above), although these mutations are genetic not physiological modulations.

Concluding remarks

The availability of genome sequence information on various microorganisms and newly developed methods of activating silent and poorly expressed genes suggest that natural product research has now entered a promising new era. Particular attention should be paid to combined approaches that include the transcription-activation of key genes and metabolism-remodeling [40]. Reinforcement of biosynthetic processes by efficient substrate supply may be synergistic with transcription-activation, eventually leading to the efficient discovery of new secondary metabolites. In support of this notion, our pilot work with the furan-like antibiotic triclosan (an inhibitor of fatty acid biosynthesis [11]) actually showed the marked efficacy of triclosan, in the genetic background of *rpoB* mutation (i.e., transcription-activation), at enhancing the actinorhodin production (nearly 3-fold enhancement) when added at low concentrations (0.1–0.5 μM) (Fig. 4). In addition, ribosome engineering can be used to activate silent biosynthetic pathways in fungi and myxobacteria. The genomes of myxobacteria are large (9.14 Mb for *Myxococcus xanthus* and 13.03 Mb for *Sorangium cellulosum*) and similar to or larger than the genome of *S. coelicolor* (8.67 Mb). Myxobacterial genomes have been found to encode many genes involved in the synthesis of secondary metabolites (e.g., 8.6 % of the *M. xanthus* genome), opening the possibility of discovering clinically relevant natural products [17, 49, 59]. Most myxobacterial metabolites are polyketides, nonribosomal peptides and hybrids of the two structures. Hence activation or enhancement of cryptic genes is of interest, and ribosome engineering may be utilized successfully for drug discovery.

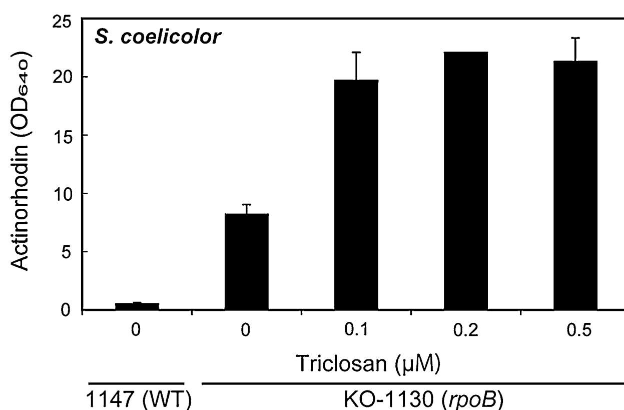


Fig. 4 Effect of exogenously added triclosan on the actinorhodin production. *S. coelicolor rpoB* (S433L) mutant strain KO-1130 was grown at 30 °C for 5–7 days in GYM liquid medium with or without triclosan. Shown are mean values of actinorhodin (OD_{640}) of triplicate flasks. Complete growth inhibition was detected at 1 μM or higher concentrations of triclosan

It is also intriguing to identify the REEs target(s) responsible for the activation of cryptic genes. As the RNAP and ribosome are both involved deeply in cryptic gene activation, REEs may bind to RNAP and/or the ribosome, eventually leading to functional alteration of these macromolecules. "Rare earth microbiology" may thus offer new insight into entirely unknown regulatory events that occur in all organisms.

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