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Activation of three natural product biosynthetic gene clusters from *Streptomyces lavendulae* CGMCC 4.1386 by a reporter-guided strategy

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ABSTRACT

Along with the fast developing of DNA sequencing technology, a great number of natural product biosynthetic gene clusters have been discovered by bioinformatic analysis, which demands novel high-throughput genome mining methods to obtain the diverse compounds dictated by those gene clusters. In this work, a method based on the reporter gene *xylE* was established to screen for the activation conditions of thirteen different gene clusters from *Streptomyces lavendulae* CGMCC 4.1386. In this reporter-guided method, the key structure gene was replaced by a *xylE-kana*^{R} cassette with the *xylE* gene being controlled by the transcription and translation machinery of the key structure gene. It not only facilitated the screening of activation conditions, but also provided the null mutants of specific natural product gene clusters as controls to link those clusters with their products conveniently. The potential activation conditions of eleven gene clusters from *S. lavendulae* CGMCC 4.1386 were obtained. In addition, activation of three of the eleven gene clusters was confirmed and their products were identified.

1. Introduction

Natural products from microbes play an important role in the field of drug discovery. In recent years, the repeated isolation of known compounds and the emergence of antibiotic resistant pathogens pose new challenges for natural product drug discovery [1,2]. With the rapid development of genome sequencing technology, more and more genomic information of microorganisms becomes available. Up to now, nearly 15,000 complete sequencing of microbial genomes have been finished according to the statistics of Genomes Online Database (www.genomesonline.org). Bioinformatic analysis of the existing data revealed that there are far more natural product gene clusters embedded in the microbial genomes than the number of the isolated compounds, indicating much larger natural product diversities are there waiting to be discovered. In addition, the studies on natural product biosynthesis in the past decades elucidated the biosynthesis mechanisms of numerous compounds and enabled the prediction of natural product gene clusters and their products' structural features, at least partially, based on the genetic information. All these progresses resulted in the development of a powerful method for natural product discovery termed genome mining. Not like the traditional bioactivity directed way to isolate natural products, in genome mining, bioinformatic analysis of the sequenced microbial genomes will predict the natural product gene clusters, which are usually silent in the ordinary culture conditions and need to be activated [3,4]. The products of those gene clusters will then be isolated, structurally determined and tested for their bioactivities.

During the genome mining studies, several different methods were used to activate the silent natural product biosynthetic gene clusters, including heterologous expression, manipulation of the regulatory genes, ribosomal engineering, cultivation in varied conditions, and so on [5,6]. There are advantages and disadvantages for each method. For example, heterologous expression and manipulation of the pathway specific regulatory genes can link the product and the gene cluster directly, but can only activate one specific gene cluster in a time; manipulation of the global regulatory genes, ribosomal engineering and cultivation in varied conditions may activated several gene clusters in a time, but it is difficult to link the metabolites with the gene clusters. For those methods toward multiple gene cluster activation, if we can find an easy way to link the gene clusters to their specific products, the throughput of genome mining may be increased significantly.

Reporter gene is a common tool used to monitor the expression status of genes, which was used successfully in silent gene cluster activation by Yang and his colleagues [7,8]. Many natural product gene clusters have tens of genes and contain several operons, which makes it

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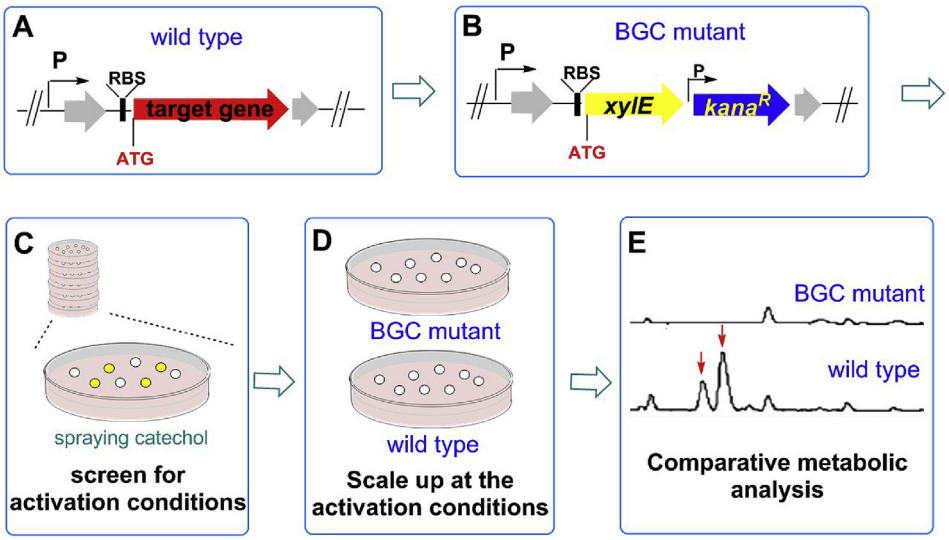

Fig. 1. Workflow of the reporter-guided genome mining strategy represented with the *xylE-kana*^{R} cassette developed in this study. (A) Analyzing a sequenced gene cluster to select the key structure gene as a target. (B) Inactivation of the key structure gene by replacing it with the *xylE-kana*^{R} cassette. The promoter, RBS and start codon of the key structure gene were kept for the reporter gene *xylE*. (C) Culture the mutants at various conditions and screen activation conditions for the expression of the reporter gene. (D) Culture the wild type and the mutant strains at the activation conditions in a large scale. (E) Comparative metabolic profiles by HPLC to identify different peaks.

Table 1
Natural product biosynthetic gene clusters from *S. lavendulae* CGMCC 4.1386.

Biosynthetic gene cluster (BGC) No.	Gene cluster type	Inactivated key structure genes (functions)	predicted product
BGC1	terpene	*orf2_168* (terpene cyclase)	squalene/hopene
BGC2	type I polyketide	*orf4_417* (ketosynthase)	unknown
BGC3	NRP	*orf5_147* (NRPS)	unknown
BGC4	terpene	*orfIV* (terpene cyclase)	geosmin
BGC5	siderophore	*orf5_45* (IucA/C, condensation and acylation)	unknown
BGC6	siderophore	*orf7_185* (IucA/C, condensation and acylation)	desferrioxamine
BGC7	siderophore	*orf8_135* (IucA/C, condensation and acylation)	unknown
BGC8	Type III polyketide	*orf8_165* (chalcone synthase)	alkylresorcinol
BGC9	type I polyketide	*orf10* (Ketosynthase)	unknown
BGC10	type II polyketide	*orf10_243* (ketosynthase)	simocyclinone
BGC11	atypical NRP	*StnN* (aminotransferase)	streptothricin
BGC12	Type I polyketide	*orf14_287* (ketosynthase)	unknown
BGC13	terpene	*orf14_188* (terpene cyclase)	unknown
BGC14	type II polyketide	*orf4_522* (ketosynthase)	landomycin
BGC15	NRP	*orf5_377* (NRPS)	unknown
BGC16	NRP	*orf8_193* (NRPS)	unknown
BGC17	other	*orf16_190* (cyclodipeptide synthase)	polysaccharide cyclodipeptide

Table 2
Activation conditions for *xylE* in different mutants.

Biosynthetic gene clusters	Activation media for the *xylE* gene	Growth stages for *xylE* activation
BGC 1	22	aerial mycelia
BGC 2	6	aerial mycelia
BGC 3	29	substrate and aerial mycelia
BGC 4	10	substrate mycelia
BGC 5	25	substrate mycelia
BGC 6	Not activated	–
BGC 7	13,42	aerial mycelia
BGC 8	Not activated	–
BGC 9	1,4,14,17,19,22,23,	substrate mycelia:1,4,17,24
	24,28,29,31,32,41	aerial mycelia: all thirteen media
BGC10	31,32	aerial mycelia
BGC11	4,10	aerial mycelia
BGC12	6,10,12	aerial mycelia
BGC13	22	aerial mycelia

difficult to monitor the transcription status of all genes in one cluster. A simple way to check the transcription status of a gene cluster is to focus on the transcription level of a key gene that is essential to the cluster's function. Only at the conditions that the selected key gene is active, the gene cluster has the possibility to be functional. For natural product biosynthetic gene clusters, the key structure genes, like genes encoding polyketide synthases (PKSs), non-ribosomal peptide synthetases (NRPSs) or terpene cyclases, are indispensable part of the gene clusters. If a specific key structure gene is replaced by a chromogenic reporter gene, which is controlled by the promoter, the ribosomal binding site (RBS) and even the start codon of the key gene for its transcription and translation, the selected conditions that can activate the expression of the reporter gene, are also conditions that should activate the key structure gene. These are also potential conditions that can activate the whole gene clusters. Meanwhile, the key structure gene mutants could be used as controls to compare with the metabolic profiles of the wild-type strain, the compounds presented in the wild type strain but absent in the mutant should be the products of the inactivated gene clusters (Fig. 1). Therefore, this reporter-guided strategy combines well the screening of activation conditions for silent gene clusters with link the products with specific gene clusters, and is potential to be used as a high-throughput genome mining method.

In this work, we constructed the null mutants of thirteen natural product gene clusters from *Streptomyces lavendulae* CGMCC 4.1386 by replacing their key structure genes with the catechol dioxygenase gene (*xylE*), a reporter gene frequently used in *Streptomyces* [9]. Specifically, the native promoters, RBSs, and start codons of those key structure genes were kept for *xylE* to ensure the expression conditions of the reporter gene can also be used to activate the corresponding gene

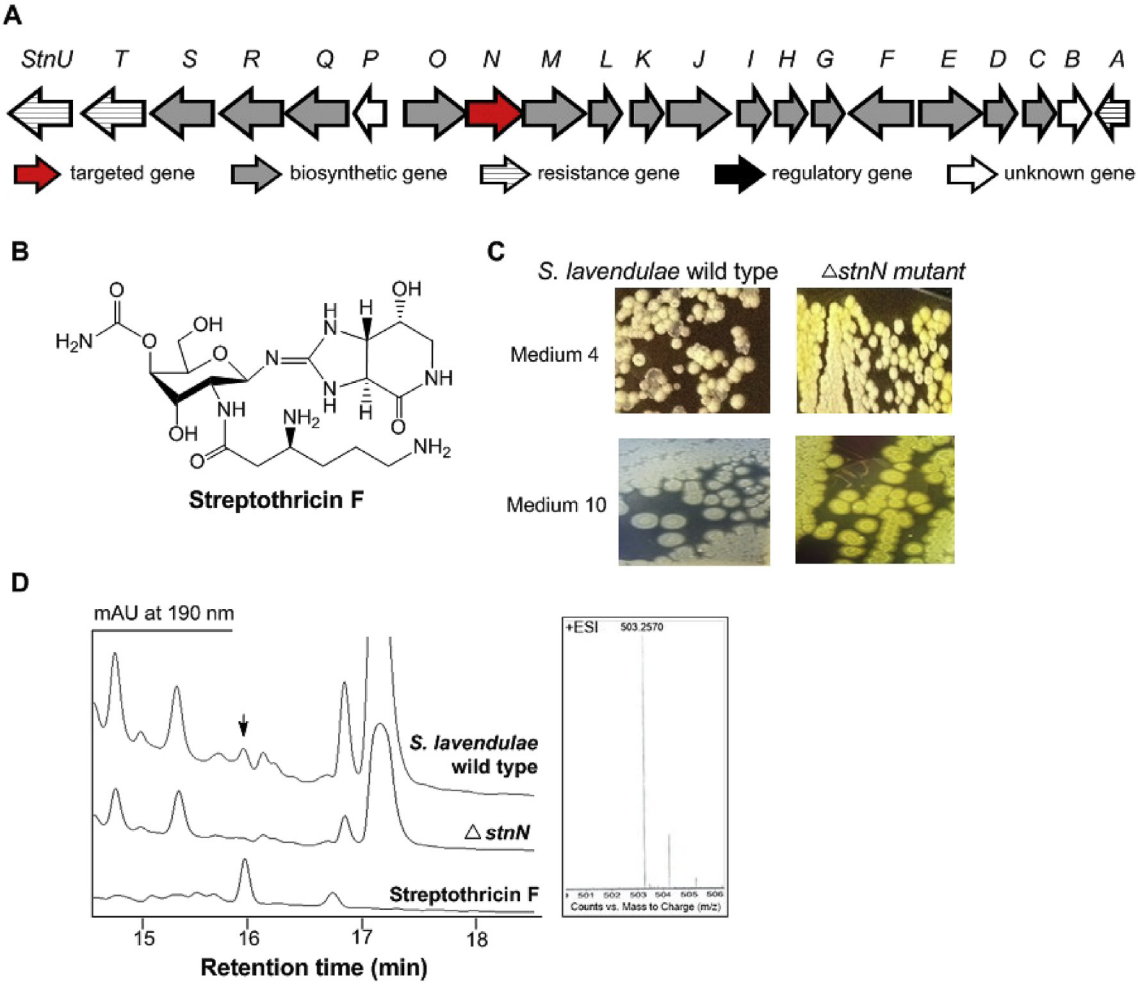

Fig. 2. Activation of the streptothricin biosynthetic gene cluster BGC11. (A) Gene organization of the streptothricin biosynthetic gene cluster BGC11. (B) Chemical structure of streptothricin F. (C) Color change of the *S. lavedulaeΔstnN* mutant on medium 4 and medium 10 after spraying of catechol. (D) Comparison of the metabolic profiles of *S. lavendulae* wild type strain and*ΔstnN* by HPLC analysis. The streptothricin F peak only appearing in *S. lavendulae* wild type is indicated with an arrow and its HR-MS spectrum was presented at right.

clusters. Activation conditions were screened by changing growth media and positive results were obtained for eleven of the thirteen mutants. At final, three gene clusters from *S. lavendulae* CGMCC 4.1386 were activated successfully and their products were identified.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

The genome sequenced *S. lavendulae* CGMCC 4.1386 [10] was analyzed and studied as a target strain for natural product discovery. *Escherichia coli* JM109 was used as a cloning host. The plasmid pDR2 and pUC119:*kana*^{R} were described previously [7,11]. Other strains and plasmids constructed in this study were listed in Table S1. Kanamycin and apramycin were added to a final concentration of 100 μg/mL and 50 μg/mL, respectively. All media used for culture condition screening were listed in Table S2.

2.2. General DNA manipulation

General DNA manipulations were performed as described [12]. PCRs were performed using the PrimeSTAR HS DNA polymerase (Takara, Shiga, Japan) according to the manufacturer's instructions. Primers used in this study are listed in Table S3. *E. coli-Streptomyces* conjugations were carried out according to the standard procedure [13].

2.3. Sequencing and bioinformatic analysis

DNA sequencing was performed in Majorbio (Shanghai, China). The open reading frame (ORF) prediction was performed with Prodigal (http://compbio.ornl.gov/prodigal/). The functional annotation combined the search results of NCBI and KEGG databases. The natural product biosynthetic gene clusters of *S. lavendulae* CGMCC 4.1386 were analyzed with antiSMASH (https://antismash.secondarymetabolites.org/).

2.4. Construction of the S. lavendulae mutants

The 0.9 kb of promoter-less *xylE* gene was PCR amplified using plasmid pDR2 as a template with primer pair xyl-F/xyl-R and cloned into the *Bam*HI/*Kpn*I sites of plasmid pUC119:*kana*^{R} to generate pUC119*xylE-kana.* Plasmid pUC119*xylE-kana* was then used as a template to PCR amplify the *xylE-kana*^{R} cassette with primer pair xk-F/xk-R. The 2.0-kb fragment containing the *xylE-kana*^{R} was digested with *Hin*dIII and *Eco*RI and cloned into the same sites of plasmid pKC1132 to generate plasmid pKC1132xk. To facilitate gene cloning, some restriction enzyme sites were introduced into plasmid pKC1132xk by the PCR primers xk-F (*Xba*I) and xk-R (*Spe*I).

The key structure gene mutants were constructed using a homologous recombination method (Fig. S1). Taken the construction of *S. lavendulaeΔstnN* as an example, two 1.9-kb fragments flanking the

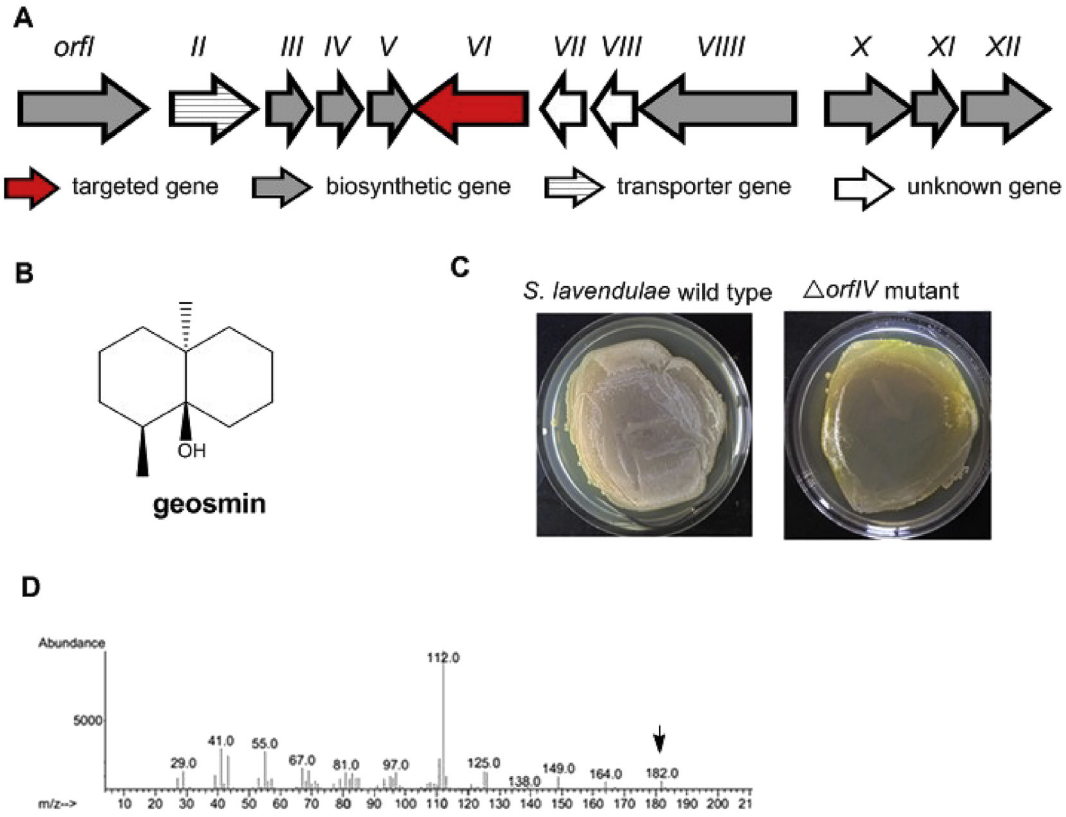

Fig. 3. Activation of the geosmin biosynthetic gene cluster BGC4. (A) Gene organization of the putative geosmin biosynthetic gene cluster. (B) Chemical structure of geosmin. (C) Color change of *S. lavendulae ΔorfIV* mutant on medium 10 after spraying of catechol. (D) The GC/MS spectrum analysis of *S. lavendulae* CGMCC 4.1386. The molecular weight of geosmin is indicated with a black arrow.

aminotransferase gene *stnN* were PCR amplified using primer paris stnNL-F/stnNL-R and stnNR-F/stnNR-R and cloned into the *Xba*I and *Spe*I sites of plasmid pKC1132xk to generate the *stnN* inactivation plasmid. This plasmid was then introduced into *S. lavendulae* CGMCC 4.1386 by E. *coli-Streptomyces*. After propagated for two generations on MS plate with kanamycin, colonies that are sensitive to apramycin but resistant to kanamycin were selected as the desired double crossover mutants *S. lavendulaeΔstnN*. The genotype of *S. lavendulaeΔstnN* was PCR confirmed with primer pair stnNver-F/stnNver-R. The other 12 key structure gene inactivated mutants were constructed in a similar manner (Fig. S1). All primers used for mutant constructions and confirmations were listed in Table S3.

2.5. Detection of the reporter gene expression

When different *S. lavendulae* mutants were cultivated on varied agar plates, 0.5 M of catechol solution were sprayed on the surface of the plates at 3, 5 and 7 days, corresponding to the substrate mycelia, aerial mycelia and sporulation stages of *S. lavendulae*, respectively. The color change of the colonies was observed after wrapped with foil and incubated for further 30 min at 28 °C. The media on which the colonies become brightly yellow were chosen as the candidate conditions for the expression of the corresponding biosynthetic gene cluster.

2.6. Metabolic profile analyses of S. lavendulae mutants

S. lavendulae CGMCC 4.1386 and varied mutants were cultured on selected solid media comparably. To analyze their metabolic profiles, agar plugs of about 500 mL solid media were taken from the plates and extracted with acetone or methanol by ultrasonication. The organic fraction was evaporated *in vacuum* and the residue was re-dissolved in 5 mL of methanol. The samples from varied mutants and the wild type strain were subjected to a reverse-phase C18 column (4.6 × 250 mm, 5 μm, Agilent, Santa Clara, CA, USA) on a Shimadzu HPLC system with DAD detector (Shimadzu, Kyoto, Japan). The column was developed with a 50 min gradient using acetonitrile and water with 0.1% trifluoroacetic acid at a flow rate of 1 mL/min. Percentage of acetonitrile was changed linearly from 5 to 100% at 0–50 min. Liquid chromatography-mass spectroscopy (LC-MS) analysis was carried out with Agilent 1200 HPLC system and 6520QTF-MS system (Agilent, Santa Clara, CA, USA) with the electrospray ionization source.

2.7. Isolation of geosmin

The mycelium of *S. lavendulae* CGMCC 4.1386 on the solid plate was collected into a round bottom flask, add about 200 mL of distilled water and heat it, the vaporized substance can be adsorbed by activated carbon, the activated carbon was then eluted with chloroform and the elute was analyzed by GC-MS with Agilent 5975C detector system and 7890 GC-MS system (Agilent, Santa Clara, CA, USA).

3. Results

3.1. In silico analysis of natural product biosynthetic gene clusters in S. lavendulae CGMCC 4.1386

S. lavendulae CGMCC 4.1386 is a known producer of streptothricin [14]. We draft sequenced the genome of *S. lavendulae* CGMCC 4.1386 in a previous work. Natural product biosynthetic gene clusters in *S. lavendulae* CGMCC 4.1386 were analyzed with antiSMASH and 17 different gene clusters encoding genes for the biosynthesis. Compounds belonging to various structural classes, including polyketides, non-ribosomal peptides (NRPs), siderophores and terpenes were discovered (Table 1).

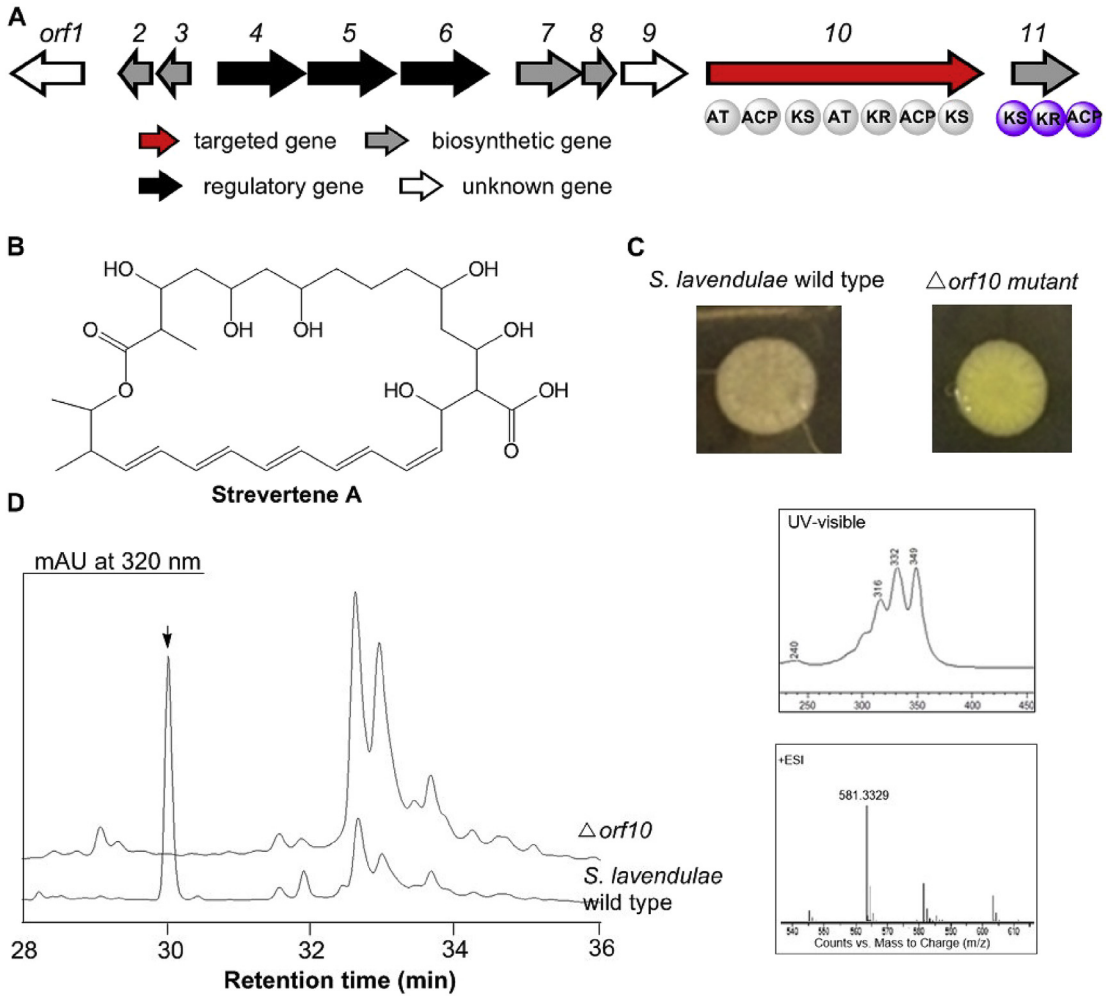

Fig. 4. Activation of the strevertene A biosynthetic gene cluster BGC9. (A) Gene organization of BGC9 and the domain representation for protein Orf10 and Orf11. (B) Chemical structure of strevertene A. (C) Color change of the *Δorf10* mutant on medium SLY after spraying of catechol. (D) Comparison of the metabolic profiles of *S. lavendulae* wild type strain and*Δorf1*0 by HPLC analysis. The peak indicated with a black arrow is presented only in *S. lavendulae* wild type strain but absent in*Δorf10* mutant.

3.2. Construction of the key structure gene inactivated mutants

To construct the key structure gene inactivated mutants of the 17 natural product biosynthetic gene clusters, the chromogenic reporter gene *xylE* and the kanamycin resistance gene *kana*^{R} were linked together by PCR to make a *xylE-kana*^{R} cassette. In this cassette, gene *kana*^{R} is controlled by its own promoter and RBS, while gene *xylE* does not have its own promoter and RBS. When the *xylE-kana*^{R} cassette was used to replace the key structure genes, e.g. the ketosynthase gene *orf10* in cluster 9, the start codon of *orf10* was used as the start codon of gene *xylE*, which implied that gene *xylE* should be controlled by the same promoter and RBS as *orf10*. The kanamycin resistance gene *kana*^{R} controlled by its own constitutive promoter was used as a selection marker that could facilitate the mutant constructions (Fig. S1). Therefore, if the yellow color generated by the catechol dioxygenase, encoded by gene *xylE*, could be observed at some specific conditions for a mutant, they were used as potential conditions for the activation of the inactivated gene cluster in this mutant (Fig. 1). Construction of the key gene inactivated mutants for all 17 natural product biosynthetic gene clusters were tried in our study. At final, the key gene inactivated mutants for cluster 1 to 13 were successfully obtained (Fig. S1).

3.3. Culture condition screening of the key structure gene inactivated mutants

Various solid media were screened for the activation conditions of the *xylE* genes in the 13 mutants. In total, 42 different media were tested for these mutants (Table S2). The substrate of catechol dioxygenase was sprayed on the surface of the solid media after grown for 3, 5 or 7 days, which were corresponding to *S. lavendulae*'s substrate mycelia, aerial mycelia and sporulation growth stages, respectively. If the *S. lavendulae* mutant colonies on a medium turned to be bright yellow after catechol was sprayed, the medium will be selected as a candidate for the activation condition of the cluster in that mutant. Eleven of the thirteen key gene inactivated mutants got positive results on at least one medium (Table 2). Some mutants seem to be much easier to be activated than the others. For example, the ketosynthase gene mutant of BGC9 turned to yellow in 13 of the 42 tested media. Notably, the *xylE* genes in 11 mutants were activated at the substrate mycelia or aerial mycelia stages, none of them was activated at the sporulation stage.

3.4. Activation of the streptothricin gene cluster

S. lavendulae CGMCC 4.1386 is a producer of streptothricin [14]. However, it was not known that whether streptothricin will be

produced on solid media, since the production conditions described for streptothricin were all in liquid media. As a proof of concept, we replaced the aminotransferase gene (*stnN*) with the *xylE-kana*^{R} cassette to construct the *S. lavendulaeΔstnN* mutant (Fig. 2A and B). It was observed that *S. lavendulaeΔstnN* turned to yellow after spraying catechol on media 4 and 10 at the aerial mycelia stage (growth for 5 days), implied that the streptothricin gene cluster was activated at the same stage on those two media (Fig. 2C). We cultured *S. lavendulaeΔstnN* and the wild type strain on media 4 and 10 at a large scale. Extracted the agar after five days growth, and subjected to HPLC analysis. The HPLC profiles of *S. lavendulaeΔstnN* and the wild type strain were scrutinized comparably to search for the peaks that were appeared in the wild type but absent in *S. lavendulaeΔstnN*. As shown in Fig. 2D, the HPLC profiles of both strains displayed a good consistency, except a peak at 15.9 min that only presented in the HPLC trace of the wild type strain. It had the same retention time with the authentic streptothricin F. The high resolution mass spectrometry (HR-MS) of this peak is 503.2570 for $[M+H]^+$ ion (calcd for $C_{19}H_{35}N_8O_8$, $[M+H]^+ = 503.2572$), supporting its identity as streptothricin F (Fig. 2D). The titer of streptothricin F was 3.8 ± 0.44 mg/L on Medium 10 agar. Successful activation of streptothricin production on solid media suggested that the reporter-guided strategy developed here can be used for natural product activation condition screening, which can also link a specific gene cluster with its products conveniently as anticipated.

3.5. Activation of the geosmin gene cluster

Geosmin is a common odor molecule produced by many *Streptomyces* strains responsible for the smell of earth [15]. A putative terpene gene cluster for geosmin biosynthesis was found in the genome of *S. lavendulae* CGMCC 4.1386 (Fig. 3A and B). The terpene cyclase gene (*orfVI*) in this cluster was replaced by the *xylE-kana*^{R} cassette to generate the mutant *S. lavendulaeΔorfVI*. It was observed that *S. lavendulaeΔorfVI* clonies turned to bright yellow on agar plate of medium 10 at the substrate mycelia stage (growth for 3 days) when sprayed with catechol (Fig. 3C). Since geosmin is a volatile substance, *S. lavendulae* CGMCC 4.1386 was cultured on medium 10 agar plates for 3 days. The agar plugs were extracted with active carbon, eluted with chloroform, and subjected to GC-MS analysis (Fig. 3D). The hint that most matched with the prepared compound by searching in the NIST library was geosmin.

3.6. Activation of a polyene gene cluster

Cluster BGC9 contains two genes encoding modular type I PKSs. Unfortunately, only partial of the gene cluster was sequenced and its product cannot be predicted by bioinformatic analysis (Fig. 4A). Significantly, the colonies of *S. lavendulaeΔorf10* turned to yellow on 13 of the 42 tested solid media, indicating that the product of this cluster is important for *S. lavendulae* in the adaption to different conditions. Medium 17 (SLY) was selected to check the activation of BGC9 in that *S. lavendulae Δorf10* was most yellow on SLY agar after spraying of catechol (Fig. 4C). A comparison of the HPLC profiles of *S. lavendulae* wild type and the *Δorf10* mutant showed that a peak at about 29.7 min was only observed in the wild type strain (Fig. 4D). The ultraviolet–visible spectrum of this peak indicated it was a polyene compound. The high resolution mass spectrometry (HR-MS) of this peak is 581.3329 for $[M+H]^+$ ion, which is identical with strevertene A ($C_{31}H_{49}O_{11}$, cacld $[M+H]^+ = 581.3320$) (Fig. 4D). The product was then analyzed by MS/MS spectrum (Fig. S2). Its fragmentation pattern confirmed its identity as strevertene A (Fig. 4B).

4. Discussion

In the post genomic era, numerous putative natural product biosynthetic gene clusters can be predicted from tons of sequencing data, which demands high-throughput genome mining methods for natural product discovery [16–19]. In this work, a method based on the reporter gene *xylE* was established to find the activation conditions of silent gene clusters. Moreover, the replacement of the key structure genes with the *xylE-kana*^{R} cassette not only facilitated the screening of activation conditions, but also provided the null mutants of specific natural product gene clusters as controls to link those clusters with their products conveniently. One advantage of this strategy is that multiple gene clusters can be screened at the same time, which serves opportunities for high-throughput natural product discovery. Just like in this case, the activation conditions of 13 gene clusters from *S. lavendulae* CGMCC 4.1386 were screened together and potential activation conditions were obtained for 11 of the 13 clusters. It should be emphasized that this reporter-guided method can only revealed the transcription and translation status of the operon that the replaced key structure gene sits in. Most of the microbial natural product gene clusters are composed of more than one operon. Therefore, the activation conditions selected by this strategy are necessary but may be inadequate for the whole gene clusters.

Only two compounds indigoidine and streptothricin were isolated from *S. lavendulae* CGMCC 4.1386 before [14]. Two more types of secondary metabolites, geosmin (terpene) and strevertene A (polyene), were successfully activated in this work, which revealed the powerfulness of this reporter-guided method as a high-throughput screening strategy. Streptothricin was known to be produced by *S. lavendulae* CGMCC 4.1386, but its production conditions on solid medium was not described before this study. Geosmin is the molecule renders soil the smell of earth. The solid medium production condition screening here implied that geosmin was produced before aerial mycelia was formed in *S. lavendulae* CGMCC 4.1386. Strevertene A, which displays inhibition activity against fungi that are detrimental to agricultural production, was isolated from *Streptoverticillium* sp. before [20]. It was shown here that *S. lavendulae* CGMCC 4.1386 was also a producer of this antibiotic. In this strain, the incomplete gene cluster BGC9 containing type I polyketide synthase encoding genes was responsible for strevertene A biosynthesis.

The repeated isolation of known compounds is still difficult to be avoided in natural product discovery, even in the genome mining efforts. Sequencing a genome completely is still expensive. Most draft sequenced genomes may contain some incomplete gene clusters, which prevent the detailed bioinformatics analyses to predict their products. In addition, only a small part of the known microbial natural products have been linked with their biosynthetic gene clusters. Therefore, repeated discovery of known compounds will happen in genome mining, even the selected gene cluster is not like any known ones. Just like strevertene A was isolated in this study. It should be noted that although strevertene A was discovered before, its biosynthetic gene cluster was never reported. The genome mining work here paves a way to study the biosynthesis mechanism of strevertene A.

In a previous study, Yang and his colleagues used *xylE* and the neomycin resistance gene in a bi-reporter guided system to screen for activation conditions of the silent *pga* gene cluster from *Streptomyces* sp. PGA64 and discovered guadimycin D [8]. Here, we developed the reporter-guided method as a high-throughput BGC activation strategy and found the possible activation conditions of 11 gene clusters from *S. lavendulae* CGMCC 4.1386. In addition, activation of 3 of the 11 gene clusters was confirmed and their products were identified. Although the reporter-guided method developed here was for *Streptomyces*, this strategy has the potential to be used in the other strains with appropriate reporter gene systems.

Conflicts of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2018.10.010.

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