Activation and comparative analysis of cryptic xiamycin gene cluster from marine-derived *Streptomyces* sp. FXJ 7.388

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Abstract: In order to obtain the natural products synthesized by the three putative xiamycin biosynthesis gene clusters which were predicted via antiSMASH during the genome mining of marine *Streptomyces* sp. FXJ 7.388, *Streptomyces* sp. FXJ 8.012, and *Streptomyces olivaceus* FXJ 7.023. Sixteen genes involved in xiamycin assembly, modification, and regulation with higher identity than the newest reported xiamycin biosynthetic gene cluster from marine *Streptomyces* sp. SCSIO 02999, *Streptomyces* sp. HKI0576, and *Streptomyces* sp. FXJ 7.388 were discovered via gene cluster comparative analysis. A ribosome engineering strategy was adopted to activate such cryptic gene clusters with different final concentrations antibiotics that act on the ribosome, and two indolosesquiterpenes were isolated from idlethaldose streptomycin-resistant *Streptomyces* sp. FXJ 7.388 strains. However, no such product was detected in *Streptomyces* sp. FXJ 8.012 and *Streptomyces olivaceus* FXJ 7.023 under the same treatment. This result suggested that these genes might hold the least gene content for xiamycin biosynthesis.

Keywords: Xiamycin; marine streptomycetes; genomic mining; biosynthesis gene cluster.

INTRODUCTION

Indolosesquiterpenes are a group of natural products isolated from plants that exhibit various activities, such as antibacterial, antiparasitic, and anti-human immunodeficiency virus (HIV), as well as inhibitory activities against lipid droplet and non-steroidal progestin biosynthesis (Yoo *et al*., 2005; Ngantchou *et al*., 2010; Kouam *et al*., 2014). For the past 10 years, an increasing number of indolosesquiterpenes have been found from fungi or actinomycetes (Ding *et al*., 2011; Li *et al*., 2012; Xu *et al*., 2012). Among these indolosesquiterpenes, xiamycin and its analogs, such as xiamycin A (**a**) and B (**b**), oxiamycin (**c**), indosespene (**d**), oridamycin A (**e**) and B (**f**), and dixiamycin C (**g**), have been isolated from *Streptomyces* species (fig. 1). Recent studies have demonstrated that the members of the xiamycin family possess different bioactivities, such as those of xiamycin A, oridamycin A, dixiamycin C, indosespene, and sespenine, against the hepatitis C virus and the herpes simplex virus-1 or selective anti-HIV activity (Sun *et al*., 2014; Meng *et al*., 2015).

Recently, the xiamycin gene cluster was cloned, identified, and characterized via genome sequencing and gene-knockout strategies from marine-derived *Streptomyces* sp. SCSIO 02999 and *Streptomyces* sp. HKI0576 (Li *et al*., 2012; Xu *et al*., 2012). During the screening program for discovering bioactive natural products from marine streptomycetes, three putative xiamycin gene clusters were predicted from marine streptomycete strains. The two xiamycin gene clusters in marine *Streptomyces* sp. FXJ 8.012 and *Streptomyces olivaceus* FXJ 7.023 displayed high similarity to xiamycin in terms of amino acid sequence, gene content, and gene order. Unlike the aforementioned two putative xiamycin

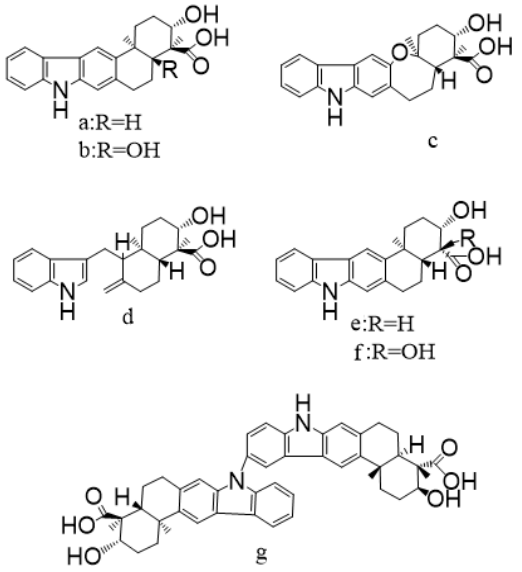

Fig. 1: Chemical structures of xiamycin (**a** and **b**), oxiamycin (**c**), indosespene (**d**), oridamycin (**e** and **f**), and dixoamycin (**g**).

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gene clusters, the xiamycin gene cluster in *Streptomyces* sp. FXJ 7.3888showed low similarity to xiamycin in terms of both amino acid sequence and gene content. A ribosome engineering technology was adopted to activate the three cryptic orphan xiamycin gene clusters, and only xiamycin A and B were isolated from idlethaldose streptomycin-resistant *Streptomyces* sp. FXJ 7.388 strains but not in *Streptomyces* sp. FXJ 8.012 and *Streptomyces olivaceus* FXJ 7.023 under the same treatment.

MATERIALS AND METHODS

Strains and reagents

Streptomyces olivaceus FXJ 7.023 and *Streptomyces* sp. FXJ 8.012 were isolated from a sediment sample collected from the South China Sea at a depth of 1108 m. Meanwhile, *Streptomyces* sp. FXJ 7.388 was isolated from a sediment sample from the shallow sea (at a depth of <1 m), which was collected from the Dalian coast of Bohai Bay. The potential of secondary metabolic and antibacterial activities of the isolated streptomycete strains are listed in table 1. The total DNA for genome sequencing and genomic library construction was extracted from the streptomycete colonies cultured in ISP 2 solid medium (Shirling *et al*., 1966). The CopyControl™ HTP Fosmid Library Production Kit (Cat. No. CCFOS059) was used to construct the genomic library were acquired from Epicentre (Madison, USA). The chemicals were purchased from the Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Oligonucleotides for polymerase chain reaction (PCR) and DNA sequencing were synthesized by Life Biotechnology Co., Ltd. (Shanghai, China). The components of the media used were obtained from BD Biosciences (Shanghai, China). The chemicals and reagents for the molecular biology and biochemistry tests were purchased from Solarbio Life Sciences (Beijing, China).

Manipulations in biochemistry and molecular biology

The methods described by Hopwood (Hopwood *et al*., 1995; Gao *et al*., 2009) were used for genomic DNA extraction of the streptomycete strains. Manipulations described by Sambrook *et al*. (2001) were performed for molecular cloning. The genomic libraries of *Streptomyces olivaceus* FXJ7.023, *Streptomyces* sp. FXJ 7.3888, and *Streptomyces* sp. FXJ 8.012 were constructed in accordance with the instructions of the manufacturer. Each library contained over 5000 clones with approximately 40 kb inserted genomic DNA fragments. DNA sequencing was performed on a 3730xl DNA analyzer at Life Biotechnology Co., Ltd. (Shanghai, China) using a shotgun cloning strategy. The primers of pCC2/pEpiFOS (including forward and reverse primers) were used to sequence the ends of each fosmid. The sequencing primers of M13F(-47) and M13R(-48) were adopted to sequence the ends of the subclones. Genome sequencing was performed on an Illumina HiSeq2000 at BGI Biotechnology Co., Ltd., (Shenzhen, China) using a proprietary reversible terminator-based strategy. The DNA sequences were uploaded to the antiSMASH website (http://antiSMASH.secondarymetabolites.org) (Weber *et al*., 2015) to determine the gene content and gene order of secondary metabolite gene clusters.

Production, isolation, and structural elucidation of xiamycin and oxiamycin

Spores were coated on ISP 2 agar medium (for *Streptomyces olivaceus* FXJ7.023 and *Streptomyces* sp. FXJ 8.012) or Difco Marine Broth 2216 (for *Streptomyces* sp. FXJ 7.3888) and a ribosome engineering strategy was adopted to activate such cryptic gene clusters with different final concentrations antibiotics that act on the ribosome (5, 10, 20, 50, and 100 ng/ml) of streptomycin, kanamycin, rifampicin, and gentamicin. Surviving colonies from sublethal concentrations of antibiotics contained in agar medium were inoculated on the same medium and selected for three generations to maintain their antibiotic resistance potential. Antibiotics-selected *Streptomyces olivaceus* FXJ7.023 and *Streptomyces* sp. FXJ 8.012 strains were cultured using a 500 ml shake flask with 100 ml ISP 2 liquid medium, for 10 d at 28 °C. An antibiotics-selected *Streptomyces* sp. FXJ 7.388 strain was cultured in VER (soluble starch, 10 g; yeast extract, 2 g; glucose, 10 g; glycerinum, 10 g; $CaCO_3$, 3 g; peptone, 5 g; corn steep powder, 2.5 g; synthetic seawater, 1000 ml), and the liquid medium was incubated at 110 rpm for 14 d at 28 °C. After cultivation, the culture was mashed and extracted thrice with 100 ml ethyl acetate. The crude extracts were applied into a silica gel column using a gradient of $CHCl_3$/MeOH to obtain the crude products after the organic portion was concentrated in vacuo to remove the solvent. A Sephadex LH-20 (MeOH) column, followed by a reverse-phase high-performance liquid chromatography column (Shimadzu SPD-M20A with Xbridge ODS 10 mm × 150 mm column), was used for further purification of the compounds. HRESI-MS (Waters Xevo G2 QTOF mass spectrometer) analyses were performed for compound identification.

RESULTS

Biosynthesis activation and chemical identification of xiamycin A and B

Over 100 antibiotics-selected colonies were collected and cultured for natural product mining, and only 1 colony derived from *Streptomyces* sp. FXJ 7.3888, namely, FXJ7.388-7K59-1, was detected with two novel products that shared an ultraviolet (UV) absorption spectrum similar to xiamycin A and B. After over 100 l fermentation broth was extracted and isolated via high-pressure liquid chromatography, two compounds were obtained (3 mg compound 1, 3 mg compound 2) from strain FXJ7.388-7K59-1 (fig. 2). The two compounds

exhibited UV absorbance and HRESI-MS spectra as follows: for compound 1, [α]D25 = −127° (c0.2, MeOH); UV λmax (MeOH:H_2O 70:30) 198, 237(sh), 260, 300 nm; HRESI-MS m/z 380.1815 (fig. 3); for compound 2, [α]D25 = −155° (c0.2, MeOH); UV λmax (MeOH:H_2O 70:30) 199, 238(sh), 261, 299 nm; HEESI-MS m/z 364.1869 (fig. 4). In accordance with these chemical data and from a biogenetic perspective, compound 1 was identified as xiamycin A and compound 2 as xiamycin B (Ding *et al*., 2011).

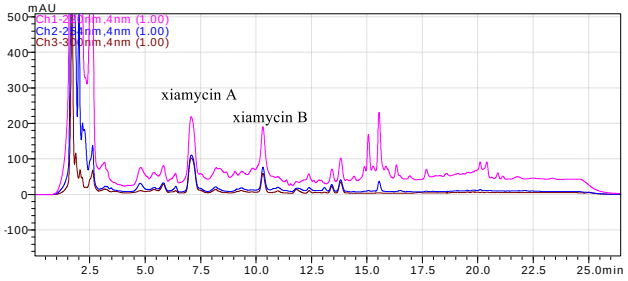

Fig. 2: Secondary metabolic spectrum of the strain *Streptomyces* sp. FXJ7.388-7K59-1.

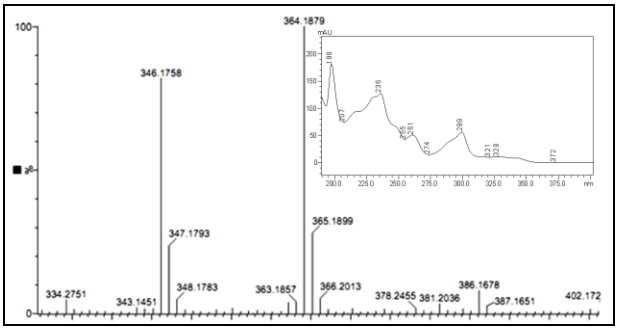

Fig. 3: MS and UV spectrum data of xiamycin A.

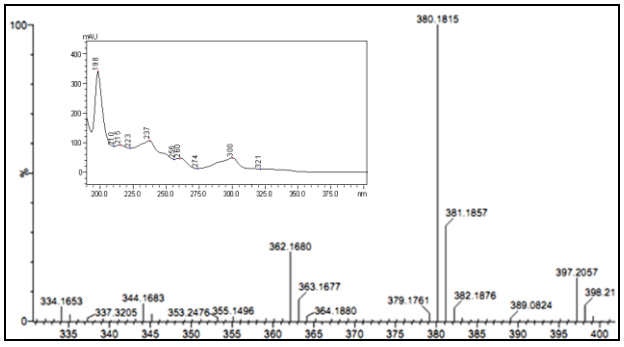

Fig. 4: MS and UV spectrum data of xiamycin B.

Sequence analysis of xiamycin gene cluster

The gene cluster online blast results of antiSMASH show that the gene clusters of xiamycin in *Streptomyces olivaceus* FXJ 7.023, *Streptomyces* sp. FXJ 8.012, and *Streptomyces* sp. FXJ 7.388 have the same gene composition, order, and orientation of the open reading frames (ORFs) with the reported gene clusters in *Streptomyces* sp. SCSIO 02999 and *Streptomyces* sp. HKI0576 for xiamycin biosynthesis. The five xiamycin gene clusters share 15 genes (Xia A to Xia O in *Streptomyces* sp. HKI0576 or Xia D to Xia R in *Streptomyces* sp. SCSIO 02999), which are involved in substrate catalysis, skeleton assembly, post-modification, and pathway regulation of xiamycin. These genes, which are found in the four xiamycin gene clusters in *Streptomyces olivaceus* FXJ 7.023, *Streptomyces* sp. FXJ 8.012 (data not shown), *Streptomyces* sp. SCSIO 02999, and *Streptomyces* sp. HKI0576, have demonstrated high identities (from 94% to 100%) in amino acid sequences and flanking genes. As an exception, the xiamycin gene cluster in *Streptomyces* sp. FXJ 7.388 exhibits lower identities (from 64% to 94%) and flanking genes than the aforementioned four clusters (fig. 5, table 2).

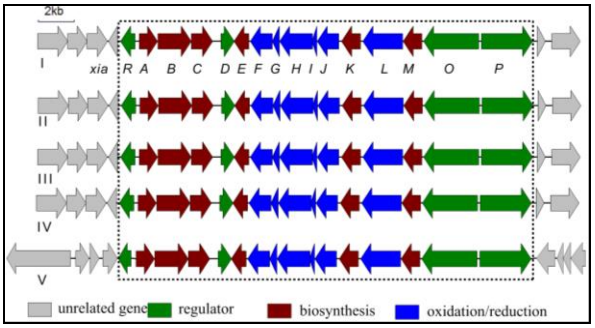

Fig. 5: Organization of xiamycin gene clusters
I: Xiamycin gene cluster from *Streptomyces* sp. SCSIO 02999,
II: Xiamycin gene cluster from *Streptomyces* sp. HKI0576,
III: Xiamycin gene cluster from *Streptomyces* sp. FXJ8.012,
IV: Xiamycin gene cluster from *Streptomyces olivaceus* FXJ7.023,
V: Xiamycin gene cluster from *Streptomyces* sp. FXJ7.388.

DISCUSSION

Two conflicting xiamycin biosynthetic pathways have been reported (Xu *et al*., 2012 and Li *et al*., 2012). In the current study, we proposed a complete putative xiamycin biosynthetic pathway based on previous works, gene cluster comparative analyses, and genome scanning in marine streptomycetes (**Fig. 6**). Over 15 enzymes are involved in xiamycin biosynthesis. Among these enzymes, 12 belong to 5 homologous gene clusters, whereas the remaining 3 are detected in the genome of *Streptomyces* sp. FXJ 7.388 but outside the xiamycin gene cluster. Five enzymes, particularly, xiaBCAKM, may be involved in the formation of the terpenoid skeleton of xiamycin (Zhang *et al*., 2015). Xiamycin biosynthesis may begins from the reaction of pyruvate and D-glyceraldehyde 3-phosphate (GAP) to form 1-deoxy-D-xylulose 5-phosphate (DXP), which is (**1**) catalyzed by Xia B (1-deoxy-D-xylulose 5-phosphate synthetase). The DXP/MEP pathway is initiated to provide isopentenyl diphosphate (IPP) (**5**) and dimethylallyl diphosphate (DMAPP) (**6**) for sequential condensation reactions that form farnesyl pyrophosphate (FPP) (7), the precursor for the biosynthesis of the sesquiterpene family (Li *et al*.,

Table 2: Comparative analysis of the xiamycin gene cluster from four *Streptomyces* strains

SCSIO 02999	FXJ7.023			FXJ7.388			HKI0576		Function regulator
Protein	Protein	Identity		Protein	Identity		Protein	Identity	
		a	b		a	b		a	
				005274					ABC membrane protein
				005275					short-chain dehydrogenase
				005276					Tet R regulator
				005277					LmbE family protein
Orf (−1)	004657	97							alcohol dehydrogenase
XiaA	004658	98							vanillate monooxygenase
XiaB	004659	99							flavin reductase
XiaC	004660	99		005278	73				IclR regulator
XiaD	004661	99	100	005279	83	83	Xia A	99	HMBPP reductase
XiaE	004662	99	99	005280	80	79	XiaB	99	DXP synthase
XiaF	004663	99	99	005281	93	94	XiaC	99	HMBPP synthase
XiaG	002664	98	100	005282	77	77	XiaD	98	LuxR family regulator
XiaH	004665	95	97	005283	75	78	XiaE	94	putative membrane protein
XiaI	004669	94	99	005285	80	79	XiaF	99	indole oxygenase
XiaJ	004666	98	100	005284	75	74	XiaG	99	limonene-1,2-epoxide hydrolase
XiaK	004667	99	100	005286	76	76	XiaH	98	aromatic ring hydroxylase
XiaL	004670	99	97	005287	66	66	XiaI	99	ferredoxin
XiaM	004672	99	100	005288	80	80	XiaJ	99	cytochrome P450
XiaN	00467	99	100	005289	77	75	XiaK	98	polyprenyl diphosphate synthase
XiaO	004673	98	99	005290	68	68	XiaL	97	monooxygenase
XiaP	004675	99	99	005291	82	82	XiaM	99	polyprenyl synthetase
XiaQ	004676	97	94	005292	69	64	XiaN	94	LuxR regulator
XiaR	004677	99	100	005293	66	66	XiaO	99	LuxR regulator
orf1	004678	98							zinc metalloprotease
orf2	004679	99							putative permease
				005294					antibiotic-resistant kinase
				005295					putative protein
				005296					XRE transcriptional regulator
				005297					DNA-binding protein

SCSIO 02999: Xiamycin gene cluster from *Streptomyces* sp. SCSIO 02999 (GenBank: JQ812811.1). HKI0576: Xiamycin gene cluster from *Streptomyces* sp. HKI0576 (GenBank: HE815466.1). FXJ7.388: Xiamycin gene cluster from *Streptomyces* sp. FXJ7.388.

2012). After the terpenoid skeleton of xiamycin of 3-farnesylindole (**8**) is formed from FPP and indole or its derivative indole-3-glycerol-phosphate by xia M, a set of tailoring enzymes, including XiaL, XiaG, XiaE, XiaJ, XiaF, and XiaH are probably involved in the modifying reactions of oxidation/reduction, cyclization, and other steps that lead to the biosynthesis of preindosespene (**11**), indosespene (**12**), xiamycin (**13**), and oxiamycin (**14**).

Compared with the gene cluster for xiamycin in *Streptomyces* sp. SCSIO 02999, there only 15 genes in the homologous gene clusters from *Streptomyces* sp. HKI0576 and *Streptomyces* sp. FXJ7.388 suggesting that the two genes of vanillate monooxygenase and flavin reductase in xiamycin gene cluster from *Streptomyces* sp. SCSIO 02999 may played few catalytic function in the biosynthesis process of xiamycin and its derivatives. The biosynthesis of xiamycin A and B were activated in the antibiotic-selected colony of *Streptomyces* sp. FXJ7.388-7K59-1 showed that ribosome engineering strategy still a powerful weapon for genomic mining of natural products.

CONCLUSION

In this study, three putative xiamycin biosynthesis gene clusters were cloned and compared from three marine-derived streptomycets. Sixteen genes may involve in xiamycin assembly, modification, and regulation via gene cluster comparative analysis. The biosynthesis of xiamycins activated by ribosome engineering strategy were and two indolosesquiterpenes were isolated from Streptomyces sp. FXJ 7.388 suggested that these genes

might hold the least gene content for xiamycin biosynthesis.

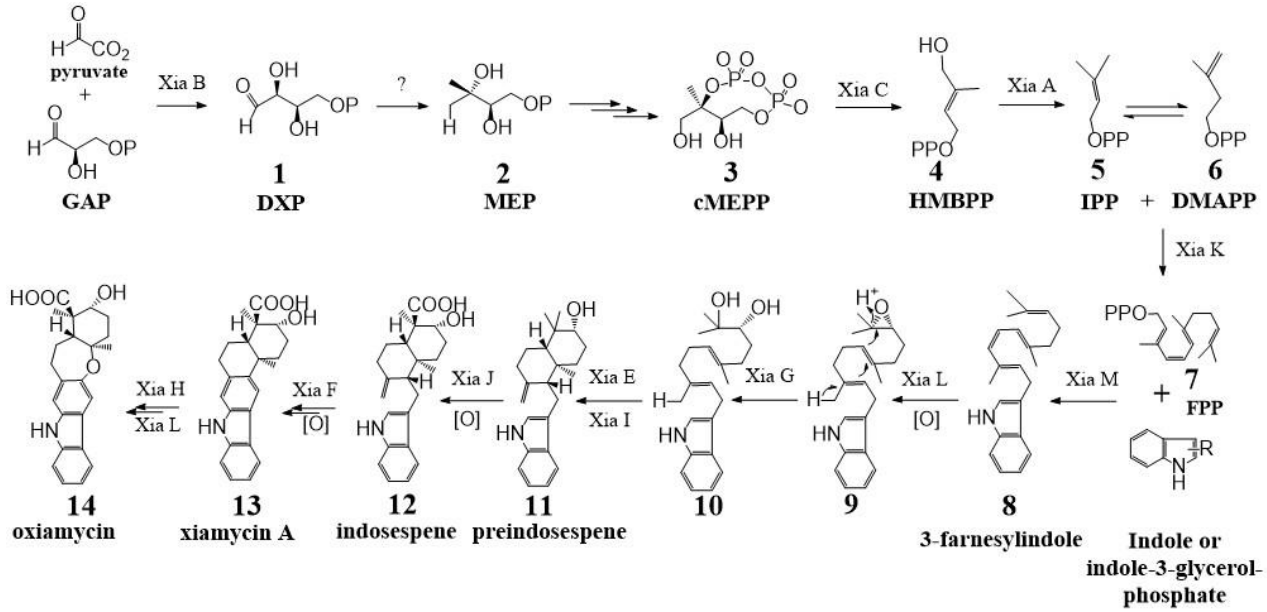

Fig. 6: Proposed xiamycin biosynthetic pathway.

ACKNOWLEDGMENTS

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