



# Genomics-guided discovery of a new and significantly better source of anticancer natural drug FK228

Xiangyang Liu<sup>a,b,1</sup>, Feng Xie<sup>c,1</sup>, Leah B. Doughty<sup>d</sup>, Qi Wang<sup>c</sup>, Lixin Zhang<sup>a,c</sup>, Xueting Liu<sup>a,\*\*</sup>, Yi-Qiang Cheng<sup>b,d,\*</sup>

<sup>a</sup> State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, 200237, PR China

<sup>b</sup> UNT System College of Pharmacy, University of North Texas Health Science Center, Fort Worth, TX, 76107, USA

<sup>c</sup> CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing, 100101, PR China

<sup>d</sup> Department of Biological Sciences, University of Wisconsin–Milwaukee, Milwaukee, WI, 53201, USA

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## ABSTRACT

FK228 is an FDA-approved anticancer drug naturally produced by *Chromobacterium violaceum* No. 968 up to 19 mg/L in a pilot industry-scale batch fermentation. Here we report a genomics-guided discovery of *Burkholderia thailandensis* MSMB43 as a new and significantly better source of FK228. The genome of *B. thailandensis* MSMB43 was found to contain a functional biosynthetic gene cluster highly homologous to that of FK228 in *C. violaceum* No. 968, and the bacterium indeed produces authentic FK228. By simple fermentation in shaking flasks in a preferred M8 medium, *B. thailandensis* MSMB43 produced FK228 up to 67.7 mg/L; by fed-batch fermentation in a 20-L fermentor in M8 medium, *B. thailandensis* MSMB43 produced FK228 up to 115.9 mg/L, which is 95 fold higher than that of *C. violaceum* No. 968 under the same laboratory fermentation conditions. RT-PCR analysis indicated that the high FK228 yield of *B. thailandensis* MSMB43 was due to high expression of biosynthetic genes, represented by *Bth\_depA*, during the fermentation process. Further genetic manipulation resulted in a recombinant strain, *B. thailandensis* MSMB43/pBMTL3-*tdpR*, which harbors a broad host-range vector expressing the thailandepsin biosynthetic pathway regulatory gene *tdpR*. This engineered strain produced up to 168.5 mg/L of FK228 in fed-batch fermentation in a 20-L fermentor in M8 medium. Therefore, the wild-type *B. thailandensis* MSMB43 or its engineered derivative could potentially be a good starting point for an industrial process to improve FK228 production for its expanding use in therapy.

## 1. Introduction

FK228 (Fig. 1B), also known as depsipeptide (FR901228), romidepsin (NSC 630176), and marketed as Istodax<sup>®</sup> by Celgene Corporation, is an anticancer drug approved by the US Food and Drug Administration (FDA) first in 2009 for the treatment of cutaneous T-cell lymphoma [1], and then in 2011 for the treatment of peripheral T-cell lymphoma [2]. It is being aggressively pursued in numerous combination therapies against various types of cancer and other disease conditions [3–7]. The bicyclic structure of FK228 is composed of four amino acids (*D*-valine, *D*-cysteine, *Z*-dehydrobutyryne, *L*-valine) and a complex moiety (3*S*,4*E*)-3-hydroxy-7-mercapto-4-heptenoic acid (HMHA), which is likely built from one *L*-cysteine unit and two C<sub>2</sub> units derived from malonyl

coenzyme A. The signature disulfide linkage in FK228 is critical for structural stability when FK228 normally exists as a prodrug, and for bioactivity as a potent histone deacetylase (HDAC) inhibitor when FK228 is reduced *in vivo* [8]. The biosynthesis of FK228 in *Chromobacterium violaceum* No. 968 was extensively studied, revealing a thio-templated, hybrid nonribosomal peptide synthetase (NRPS)-polyketide synthase (PKS) assembly line with multiple accessory enzymes and a stress-inducible transcriptional regulator [9–13].

Chemical synthesis of FK228 has been difficult due to challenges of asymmetric construction of the HMHA moiety, the formation of a 16-membered cyclic depsipeptide, and intramolecular oxidative coupling of two thiol moieties to form a 15-membered disulfide-containing ring [14,15]. Current commercial supply of FK228 is made possible through

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\* Corresponding author. UNT System College of Pharmacy, University of North Texas Health Science Center, Fort Worth, TX 76107, USA.

\*\* Corresponding author. State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, 200237, PR China

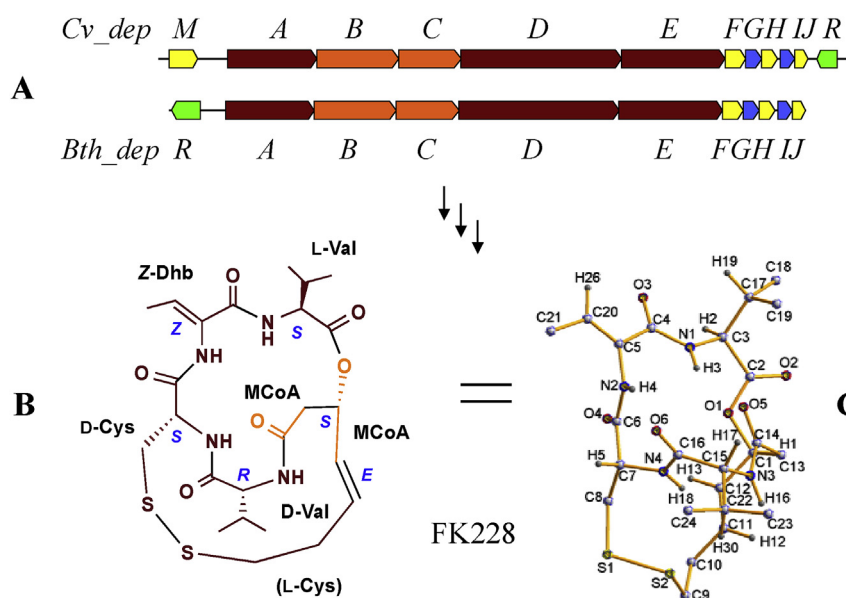
E-mail addresses: [Liuxt2010@126.com](mailto:Liuxt2010@126.com) (X. Liu), [YiQiang.Cheng@unthsc.edu](mailto:YiQiang.Cheng@unthsc.edu) (Y.-Q. Cheng).

<sup>1</sup> These two authors made equal contribution to the work.

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**Fig. 1.** FK228 biosynthesis cluster and structure. (A) A graphic comparison between two highly homologous FK228 biosynthetic gene clusters from *C. violaceum* No. 968 (*Cv\_dep*) and *B. thailandensis* MSMB43 (*Bth\_dep*), respectively. Genes are color-coded to indicate functional categories of their encoded proteins. Dark red: nonribosomal peptide synthetases; orange: polyketide synthetases; yellow: tailoring proteins; blue: resistance proteins; green: regulatory proteins. (B) A 2-dimensional structure of FK228, with molecular building blocks labeled. (C) A 3-dimensional molecular drawing of FK228.

microbial fermentation of *C. violaceum* No. 968, although the exact market size of Istodax® and the yield of industrial production and conditions of FK228 are proprietary [16]. An original report of FK228 production up to 19 mg/L from a pilot industry-scale of bacterial fermentation was achieved [17].

*Burkholderia* species have been shown to be a productive source of diverse natural products [18], and multiple laboratories have isolated unique depsipeptides related to FK228 including burkholdacs [19], FR901375 [20,21], spiruchostatins [22,23] and thailandepsins [24,25]. In this paper, we report the discovery of a biosynthetic gene cluster in the genome of *B. thailandensis* MSMB43 which is highly homologous to that of FK228 in *C. violaceum* No. 968, and show that *B. thailandensis* MSMB43 produces a high titer of authentic FK228 in a defined growth medium through laboratory-scale fermentation. We suggest that *B. thailandensis* MSMB43 wild-type strain, or an engineered derivative of which, could potentially be a good starting point for an industrial process to improve FK228 production for its expanding use in therapy.

## 2. Materials and methods

### 2.1. Bacterial strains and genomic analysis

The bacterial strains and plasmids used in this study are listed in Table S1 (Supplemental Material). The original draft genome sequence of *B. thailandensis* MSMB43 was reported by T. D. Read and associates in 2010 [26], but the quality was found to be suboptimal for our natural product discovery purpose due to insufficient coverage. We thus re-sequenced the bacterial genome and obtained a significantly improved draft genome sequence, which contains at least 13 secondary metabolite biosynthetic gene clusters [27]. Sequence gaps within a targeted gene cluster were filled by nested PCR and Sanger sequencing [28]. Search for homologous gene and protein sequences was performed with Blast algorithms available at the NCBI server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi/>) [29]. Analysis of the domain and module organization of PKSs and NRPS was carried out with popular web servers [30,31].

### 2.2. Genetic manipulation of *B. thailandensis* MSMB43

To create a series of *B. thailandensis* MSMB43 derivative strains (Table S1), vector pBMTL-3 and three gene-expression constructs [pBMTL3-*depR*, pBMTL3-*spiR* and pBMTL3-*tdpR*] were introduced into *B. thailandensis* MSMB43 cells via interspecies conjugation as follows. Donor strain *E. coli* S17-1/vector or construct was grown in Luria-

Bertani (LB) media supplemented with 10 µg/mL tetracycline (Tc<sup>10</sup>) and 34 µg/mL chloramphenicol (Cm<sup>34</sup>) at 37 °C with shaking until the late mid-log phase (6–8 h); *B. thailandensis* MSMB43 was grown similarly with 50 µg/mL apramycin (Am<sup>50</sup>) at 30 °C until the late mid-log phase (6–8 h). Cells from 1 mL of each culture were collected by centrifugation at 4,000 × g for 15 min at 4 °C, and the cell pellets were washed once with 1 mL LB medium. Cells were collected again by centrifugation and resuspended in 100 µL LB medium. Cell suspensions of two bacterial strains were pooled and spread evenly on a wet 0.45-µm nitrocellulose membrane (GE Healthcare, Pittsburgh, PA) on LB agar. After the plate had been incubated at 30 °C for 12–16 h, the membrane seeded with bacteria was used to print several LB agar plates containing Am<sup>50</sup> and Cm<sup>50</sup> to select for exconjugants.

### 2.3. Real-time quantitative PCR

*B. thailandensis* MSMB43 and *C. violaceum* No. 968 were grown for 48 or 24 h in LB broth as seed cultures, respectively. Then 1.6 mL of each seed culture was inoculated into 40 mL M8 medium for 72 h fermentation. Aliquots of culture were collected for RNA extraction, and the remaining cultures were used for quantification of the FK228 production levels of the two strains. Extraction of total RNA was performed using AxyPrep Multisource Total RNA Miniprep kit (AxyGen, CA, USA). RNA samples were digested with DNase I (Takara, Dalian, China) to remove residual DNA and then recovered using RNA Clean kit (Tiangen, Beijing, China). Absence of DNA contamination in RNA samples was confirmed with conventional PCR reactions. DNA-free RNA samples were then reverse transcribed to cDNA using reverse transcriptase M-MLV kit (Takara). Genes *depA*, *depJ* and *depR* were chosen for real-time PCR; their primer sequences were listed in Table S2. Real-time PCR was performed using SYBR Premix Ex Taq kit (Tiangen). A typical reaction of 20 µL contains 10 µL 2X Premix Ex Taq DNA polymerase, 4 pmol of each primer and 100-fold dilution (for 16s rDNA) or 10-fold dilution (for other genes) cDNA solution. PCR program was as followed: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 62 °C for 31 s. The expression levels of target genes were first normalized to reference 16s rDNA gene. Then, fold differences between the expression levels of genes in *B. thailandensis* MSMB43 relative to their counterparts in *C. violaceum* No. 968 were calculated using 2<sup>-ΔΔCt</sup> method [32].

### 2.4. Bacterial fermentation

For small volume fermentation in shaking flasks, a seed culture of

**Table 1**A comparison of genes and deduced proteins between homologous FK228 biosynthetic (*dep*) gene clusters in two bacterial species.

<i>dep</i> Gene Cluster in <i>C. violaceum</i> No. 968		<i>dep</i> Gene Cluster in <i>B. thailandensis</i> MSMB43		Gene sequence identity/ gap	Protein sequence similarity/identity	Confirmed or deduced protein function
<i>Gene</i>	Deduced protein (# of amino acids)	<i>Gene</i>	Deduced protein (# of amino acids)			
<i>Cv_depM</i>	<i>Cv_DepM</i> (389)	–	–	–	–	Aminotransferase
–	–	<i>Bth_depR</i>	<i>Bth_DepR</i> (442)	–	–	AraC-type transcriptional regulator
<i>Cv_depA</i>	<i>Cv_DepA</i> (1,697)	<i>Bth_depA</i>	<i>Bth_DepA</i> (1,694)	79%/2%	77%/85%	NRPS <sup>a</sup>
<i>Cv_depB</i>	<i>Cv_DepB</i> (1,553)	<i>Bth_depB</i>	<i>Bth_DepB</i> (1,563)	84%/1%	84%/89%	PKS <sup>b</sup>
<i>Cv_depC</i>	<i>Cv_DepC</i> (1,183)	<i>Bth_depC</i>	<i>Bth_DepC</i> (1,187)	83%/1%	82%/88%	PKS <sup>b</sup>
<i>Cv_depD</i>	<i>Cv_DepD</i> (3,057)	<i>Bth_depD</i>	<i>Bth_DepD</i> (3,081)	81%/2%	82%/87%	NRPS <sup>a</sup>
<i>Cv_depE</i>	<i>Cv_DepE</i> (1,892)	<i>Bth_depE</i>	<i>Bth_DepE</i> (1,923)	82%/2%	80%/86%	NRPS <sup>a</sup>
<i>Cv_depF</i>	<i>Cv_DepF</i> (390)	<i>Bth_depF</i>	<i>Bth_DepF</i> (390)	88%/ < 1%	93%/96%	FadE2-like acyl-CoA dehydrogenase
<i>Cv_depG</i>	<i>Cv_DepG</i> (321)	<i>Bth_depG</i>	<i>Bth_DepG</i> (321)	82%/ < 1%	86%/92%	Phosphotransferase
<i>Cv_depH</i>	<i>Cv_DepH</i> (319)	<i>Bth_depH</i>	<i>Bth_DepH</i> (301)	86%/1%	86%/91%	FAD-dependent disulfide oxidoreductase
<i>Cv_depI</i>	<i>Cv_DepI</i> (304)	<i>Bth_depI</i>	<i>Bth_DepI</i> (304)	81%/2%	81%/88%	Esterase/Lipase
<i>Cv_depJ</i>	<i>Cv_DepJ</i> (254)	<i>Bth_depJ</i>	<i>Bth_DepJ</i> (266)	72%/1%	67%/79%	Type II thioesterase
<i>Cv_depR</i>	<i>Cv_DepR</i> (312)	–	–	–	–	OxyR-type transcriptional regulator

NRPS.

–: not available.

<sup>a</sup> : nonribosomal peptide synthetase; PKS.<sup>b</sup> Polyketide synthase.

50 mL of *B. thailandensis* MSMB43 in LB medium containing Am<sup>50</sup>, or *C. violaceum* No. 968 in 1% Difco nutrient broth (NB) containing 200 µg/mL of ampicillin (Ap<sup>200</sup>), was cultivated at 30 °C for 24 h with agitation (200 rpm). Subsequently, 10 mL seed culture of each strain was inoculated into 300 mL NB/G medium (NB with 1% glucose; pH 7.0; systematically named as M1 medium) or M8 medium (0.5% glucose, 0.5% peptone, 0.3% NaCl, 0.12% Na<sub>2</sub>HPO<sub>4</sub>, and 0.05% KH<sub>2</sub>PO<sub>4</sub>; pH 7.0) in each of four 1-L flasks, and cultured at 30 °C for 120 h with agitation (150 rpm). Similarly for large volume batch fermentation, 250 mL seed culture was inoculated into 12 L M8 medium in each of two 20-L fermentors (BioFlo IV, New Brunswick Scientific Co., NJ) and fermented at 30 °C for 120 h under 20 L/min aeration and 200 rpm agitation; pH was maintained at 7.0 with 1 N NaCl or 1 M HCl. For large volume fed-batch fermentation, 3 L of 10X concentrated medium was fed between 24 h and 48 h on top of the batch fermentation. Fermentation of genetically altered *B. thailandensis* derivative strains was conducted similarly but with the addition of Cm<sup>50</sup> to the medium to maintain the pBMTL-3 based plasmids. Furthermore, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the medium at 0 h and again at 48 h to a final concentration of 0.5 mM to induce gene expression.

## 2.5. Quantification of FK228 production by liquid chromatography-mass spectrometry (LC-MS)

All LC-MS analyses were performed on an Agilent 1100 series LC/MSD Trap SL mass spectrometer equipped with an Agilent Zorbax ODS C18 column (3.0 × 10 mm) and a UV detector. The solvent system consists of buffer A (water with 0.1% formic acid) and buffer B (acetonitrile with 0.1% formic acid). The LC program included a linear gradient from 40% buffer B to 100% buffer B in 10 min, a constant elution in 100% buffer B for 2 min, followed by a linear return to 40% of buffer B in 1 min. Flow rate was 0.5 mL/min and UV spectrum was monitored at 210 nm. The inert nitrogen gas flow rate, nebulizer pressure and drying temperature for the MS system were 10 L/min, 30 psi and 350 °C, respectively. MS was scanned in positive mode in a range of 100 to 1000 *m/z*; the target compound FK228 (540.1 *m/z*) was eluted at 3.6–4.5 min under those conditions. A standard curve of FK228 concentrations vs. ion signal peak areas was first generated by running a series of authentic FK228 solutions in acetonitrile (0.5, 1, 2, 4, 6, 8 and 10 µg/mL) on LC-MS in triplicates, and the mean value of

peak areas of extracted FK228 ion signal were plotted against the concentrations (Fig. S1). The standard curve showed a good linear correlation between varying concentrations and peak areas of the extracted ion signal of FK228. Fermentation cultures (10 mL from each of the four flasks and then pooled together, or 40 mL from a fermentor) were sampled at 24, 48, 72, 96 and 120 h, respectively, and extracted three times with equal volume of ethyl acetate. Three extracts were pooled and concentrated to dryness with a rotary evaporator, and the resulting residue was dissolved in 1 mL of acetonitrile. This suspension was centrifuged at 8000 rpm for 3 min, and 10 µL of the supernatant or proper dilutions was subjected to LC-MS analysis as described above.

## 2.6. Isolation of FK228 from larger scale fermentation

Because FK228 was previously found to present mostly in the aqueous phase, fermentation broth was first centrifuged at 3800 rpm for 20 min to remove cell debris. The resulting supernatant was passed three times through a large glass column (i.d. 10 × 100 cm) packed 1/3 full with a mixture of Amberlite XAD16 resin (20–60 mesh, Sigma-Aldrich, USA) and Diaion HP-20 resin (Sigma-Aldrich) in a 1:1 ratio. Resin mixture was further washed with water, drained and eluted repeatedly with three volumes of ethyl acetate. Elution fractions were pooled and concentrated with a rotary evaporator, and the resulting oily residue was subjected to three steps of chromatographic separation to obtain FK228 with greater than 98% purity (Fig. S2).

## 2.7. Nucleotide sequence accession number

The nucleotide sequence of the newly identified FK228 biosynthetic gene cluster from *B. thailandensis* MSMB43 (*Bth\_dep*) reported in this paper has been deposited in the GenBank database under accession number JX268530.

## 3. Results

### 3.1. *B. thailandensis* MSMB43 possesses a putative FK228 biosynthetic gene cluster (*Bth\_dep*)

Bioinformatic analysis of the 7.2-Mb improved draft genome sequence of *B. thailandensis* MSMB43 revealed at least 13 putative gene clusters coding for the biosynthesis of a wide range of natural products

[27]. Among them, one gene cluster (No. 13; annotated as *Bth\_dep*) is highly homologous to that of FK228 from *C. violaceum* No. 968 (labeled in this paper as *Cv\_dep* for distinguishing from *Bth\_dep*; GenBank accession no. EF210776) [9–12] (Fig. 1; Table 1). There are only two points of difference between these two gene clusters. First, *depM* is present in the *Cv\_dep* gene cluster but not in the *Bth\_dep* gene cluster; it is a relevant but nonessential gene encoding a putative aminotransferase for FK228 biosynthesis in *C. violaceum* No. 968 [12]. Second, *Cv\_depR* encodes an OxyR-type transcriptional regulator and is located at the far right of the *Cv\_dep* gene cluster, while *Bth\_depR* encodes an AraC-type transcriptional regulator and is located at the far left of the *Bth\_dep* gene cluster; these two regulatory genes or their deduced proteins do not have significant sequence homology. The remaining genes (*depABCDEFGHIJ*) in the two gene clusters are overwhelmingly homologous in terms of gene order, gene size, sequence at either nucleotide level or deduced amino acid level, and the predicted substrate specificity of NRPS or PKS enzymes. Therefore, it was conveniently assumed that, if this new *Bth\_dep* gene cluster is functional and expressed, its translated hybrid NRPS-PKS assembly line with multiple accessory enzymes shall direct the biosynthesis of FK228 in *B. thailandensis* MSMB43.

### 3.2. Identification of cultivation conditions for *Bth\_dep* gene cluster expression and FK228 production

*B. thailandensis* MSMB43 was grown in a set of nine media previously described [24]. RT-PCR analysis showed that *Bth\_depJ*, which is the last gene of the *Bth\_dep* gene cluster and represents the gene cluster, is abundantly expressed in M1, M7 and M8 media, but not at all in M2, M3, M6 or M11 medium at 48 h of growth (Fig. 2A). Subsequently, comparative analysis of the ethyl acetate extracts of *B. thailandensis* MSMB43 cultures grown in the nine media by HPLC revealed a well-separated peak present in the extract of M8 culture but absent in that of M11 culture (Fig. 2B; M8 and M11 profiles chosen as examples for their clean background around the target peak). This peak was collected, vacuum-dried, and the material of which was subjected to LC-MS analysis, revealing a dominant compound with an ion signal of  $m/z$  540.1 for  $[M + H]^+$  (Fig. 2B insert), consistent with our previous detection of FK228 with the same instrument under the same experimental conditions [9]. Further large-scale purification from the M8 culture afforded a batch of FK228 with greater than 98% purity. The

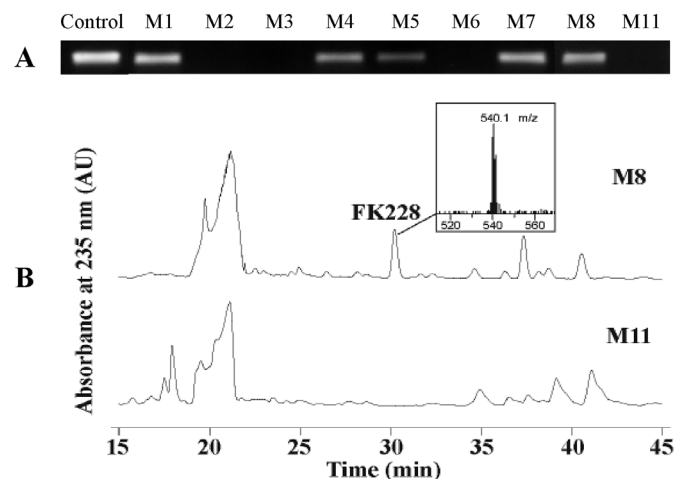


Fig. 2. Differential gene expression and FK228 production. (A) RT-PCR profiling of *Bth\_depJ* (representing *Bth\_dep* gene cluster) expression in nine different media. Amplification of a 283-bp DNA from genomic template was included as a positive control. (B) HPLC profiling of FK228 productivity in M8 and M11 media with UV absorbance monitored at 235 nm. Insert shows an LC-MS chromatography of FK228 with  $[M + H]^+ = 540.1 m/z$ .

chemical identity and structure of FK228 (Fig. 1B) was confirmed by  $^1H$ ,  $^{13}C$ ,  $^1H$ - $^1H$  COSY,  $^1H$ - $^{13}C$  HSQC, and  $^1H$ - $^{13}C$  HMBC NMR analyses (see respective  $^1H$  and  $^{13}C$  NMR spectra in Fig. S3 and Fig. S4). The extracted NMR data (Table S3) match to the originally reported NMR data of FK228 [33]. The absolute configuration of purified FK228 was previously shown by X-ray crystallography (Fig. 1C) [34] to be identical to that of the originally reported FK228 [33]. The *Bth\_dep* gene cluster was thus concluded to be functional and *B. thailandensis* MSMB43 was found to be a new source of FK228.

### 3.3. Identification of M8 as an optimal medium for FK228 production

The original FK228-producer, *C. violaceum* No. 968, and the newly identified FK228-producer, *B. thailandensis* MSMB43, were cultivated side by side in M1 medium [the original NB/G medium [17]] and M8 medium in flasks for 120 h. The time-course of FK228 production by two strains in two media was assessed by LC-MS. It was found that both strains produced higher titers of FK228 in M8 medium than in M1 medium and the maximal FK228 productivity of both strains in M8 medium was achieved between 72 h and 96 h. The measured highest titer of FK228 produced by *B. thailandensis* MSMB43 in M8 medium at 72 h was 67.7 mg/L, which is strikingly 135-fold higher than the highest titer by *C. violaceum* No. 968 at 96 h (0.5 mg/L) (Fig. 3). M8 was thus selected as an optimal medium for further FK228 productivity investigations.

### 3.4. Improved FK228 production in a fed-batch fermentation

Both *C. violaceum* No. 968 strain and *B. thailandensis* MSMB43 strain were first batch-fermented in M8 medium side by side in two 20-L fermentors. The FK228 productivity of *B. thailandensis* MSMB43 reached the highest titer of 30.2 mg/L at 48 h, and that of *C. violaceum* No. 968 reached the highest titer of 0.8 mg/L at 24 h (Fig. 4A). Biomass quantification showed that *C. violaceum* No. 968 culture had already reached its maximal growth at 24 h, while *B. thailandensis* MSMB43 at 48 h (Fig. 4B), suggesting a rather quick depletion of limited nutrients from the medium. For this reason, fed-batch fermentation was subsequently performed for these two strains. As a result, the FK228 productivity of *B. thailandensis* MSMB43 reached the highest titer of 115.9 mg/L at 96 h, and that of *C. violaceum* No. 968 reached the highest titer of 1.2 mg/L at 96 h as well (Fig. 4C). There is a 96-fold yield difference of FK228 between the two strains. Biomass quantification showed that both cultures reached their maximal growth at 72 h (Fig. 4D), indicating that nutrient replenishment is critical for achieving higher FK228 titers. *B. thailandensis* MSMB43 was thus proven to be a

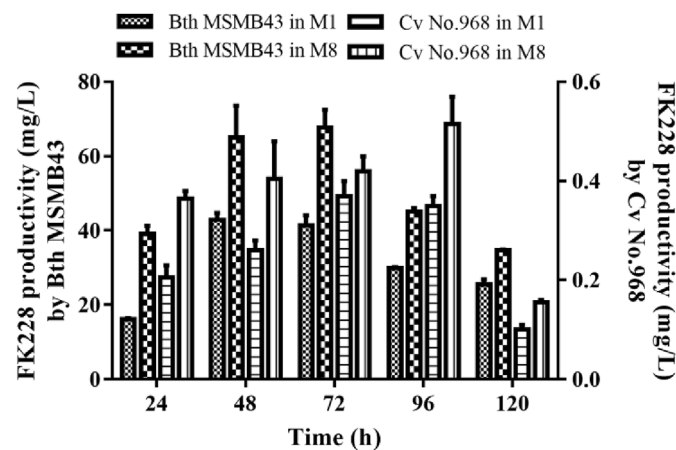


Fig. 3. Time-course of FK228 production by *B. thailandensis* MSMB43 and *C. violaceum* No. 968 in M1 or M8 medium in small volume fermentation in shaking flasks.

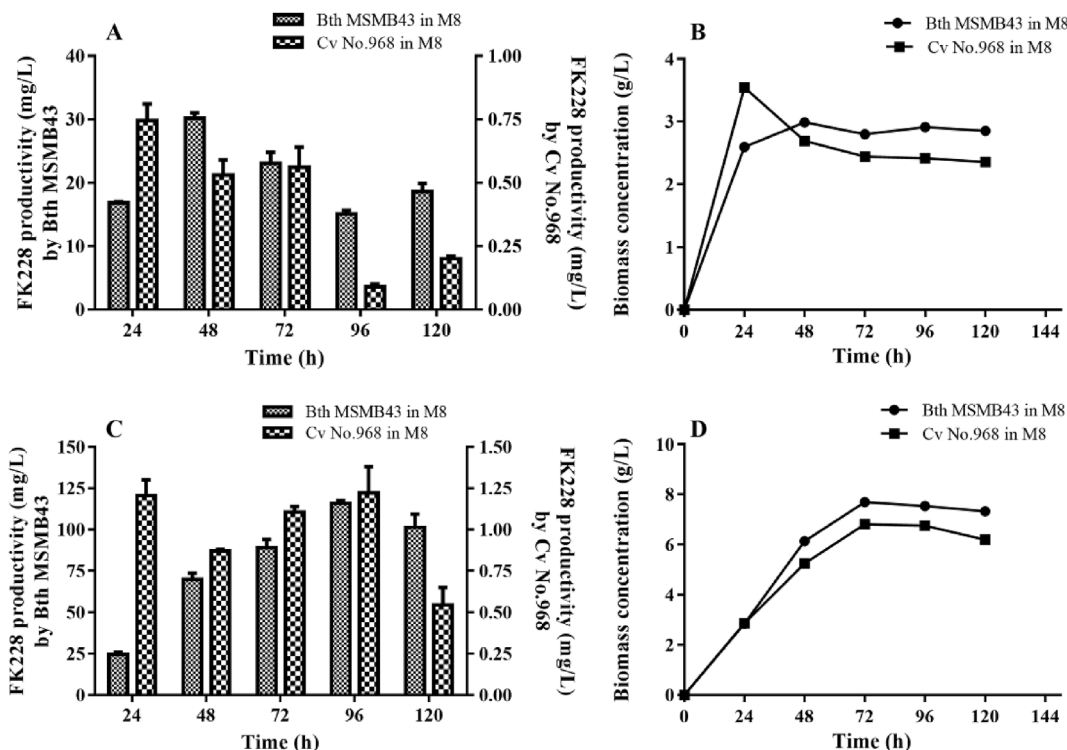


Fig. 4. Time-course of FK228 production and biomass concentrations by *B. thailandensis* MSMB43 and *C. violaceum* No. 968 in M8 medium by fermentation in laboratory-scale fermentors. (A) FK228 production in batch fermentation; (B) biomass concentrations in batch fermentation; (C) FK228 production in fed-batch fermentation; (D) biomass concentrations in fed-batch fermentation.

significantly better source of FK228.

### 3.5. High expression of *depA* in *B. thailandensis* MSMB43

To probe the underlining mechanism for a high yield of FK228 production in *B. thailandensis* MSMB43, real-time PCR was performed to exam the expression of *depA* (representing the first committed biosynthetic gene of the gene cluster), *depJ* (representing the last committed tailoring gene of the gene cluster) and *depR* in both strains cultivated in M8 medium (Fig. 5). It revealed that the relative level of *depA* expression in *B. thailandensis* MSMB43 is about 164 fold higher than that in *C. violaceum* No. 968, which is consistent with the greatest difference

of FK228 yield between these two strains grown in flasks in M8 (Fig. 3). For *depJ*, the relative expression level is only about 4.8 fold difference, suggesting that tailoring genes are not so critical for the high production of FK228 in *B. thailandensis* MSMB43. Surprisingly, the relative expression level of *depR* in *B. thailandensis* MSMB43 is only a fraction (0.19 fold) of that in *C. violaceum* No. 968, suggesting that either *Bth\_depR* in *B. thailandensis* MSMB43 is much more efficient than *Cv\_depR* in *C. violaceum* No. 968, or more likely these two regulatory genes have a totally different mode of regulating downstream biosynthetic genes.

### 3.6. Further FK228 yield improvement through metabolic engineering

Because pathway regulatory genes often play a critical role in natural product biosynthesis, yet they are small and amenable to genetic manipulation, we tested whether overexpression of a heterologous regulatory gene in *B. thailandensis* MSMB43 have an impact on FK228 production. Three regulatory genes—*depR* from the FK228 biosynthetic gene cluster in *C. violaceum* No. 968 [12], *spiR* from the spiruchostatin biosynthetic gene cluster in *Pseudomonas* sp. Q71576 [35] and *tdpR* from the thailandepsin biosynthetic gene cluster in *B. thailandensis* E264 [24]—were cloned into pBMTL-3, a broad host-range expression vector [36], the resultant constructs—pBMTL3-*depR*, pBMTL3-*spiR* and pBMTL3-*tdpR*, along with vector control—were independently introduced into *B. thailandensis* MSMB43 via conjugation to create recombinant strains *Bth* MSMB43/pBMTL3-*depR*, -*spiR* or -*tdpR*, and a control strain *Bth* MSMB43/pBMTL-3 (Table S1). Small volume fermentation in flasks in M8 medium showed that strain *Bth* MSMB43/pBMTL3-*tdpR* produced 155.4 mg/L FK228, the highest amount at 48 h (Fig. 6A). Time-course production of FK228 of this recombinant strain in fed-batch fermentation in M8 showed that FK228 reached the highest value of 168.5 mg/L at 48 h (Fig. 6B), indicating that genetic manipulation of metabolic pathway expression is effective in further improving the yield of FK228.

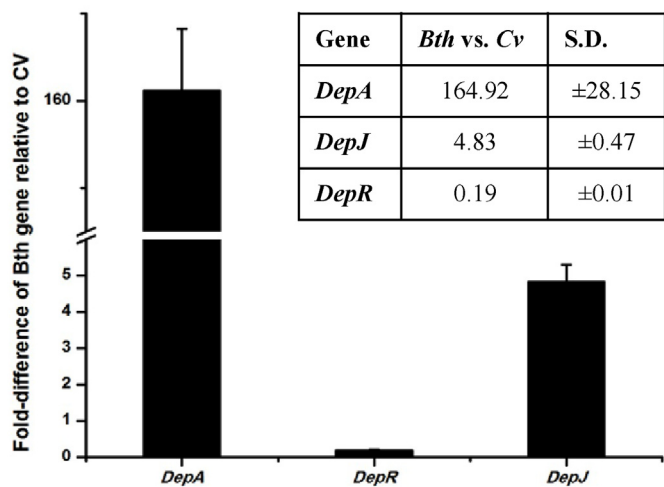
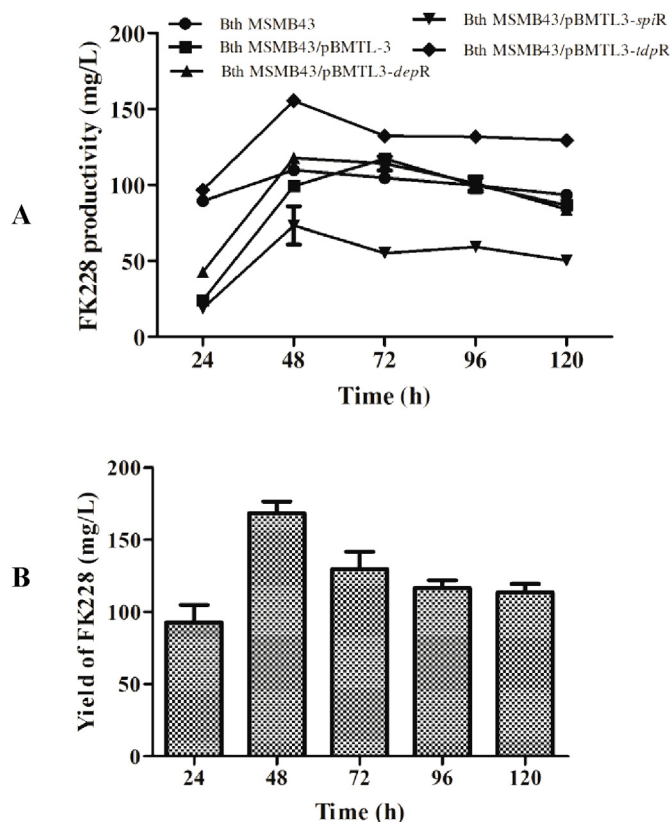


Fig. 5. Quantification by real-time PCR of relative gene expression levels (*B. thailandensis* MSMB43 vs. *C. violaceum* No. 968). Data are mean values of results from triplicate experiments, with error bars indicating standard deviation.



**Fig. 6.** Yield improvement of FK228 through genetic manipulation. (A) Time-course of FK228 production by *B. thailandensis* MSMB43 wild type and derivative strains in M8 medium in small volume fermentation in shaking flasks. (B) Time-course of FK228 production by *B. thailandensis* MSMB43/pBMTL3-*tdpR* strain in M8 medium by fermentation in laboratory-scale fermentor.

## 4. Discussion

### 4.1. A new and significantly better source of FK228

Potent microbial natural products are usually produced in minute amount, which often creates a bottleneck issue in drug development process. FK228 was discovered in early 1990s from the culture broth of *C. violaceum* No. 968, and the reported best yield was 19 mg/L by a pilot-scale industrial process [17]. As FK228 had forged its way into clinics as a new class of drug for the treatment of multiple forms of T-cell lymphoma [1,2] and potentially other types of neoplasm through combination therapies, material supply for drug manufacturing may become a limiting factor. Previously we had tapped into the FK228 biosynthetic potential, achieved moderate 10%–34% yield increase of FK228 in *C. violaceum* No. 968 through overexpressing either the *Cv\_depR* gene encoding a transcriptional regulator or the *Cv\_depH* gene encoding a rate-limiting disulfide oxidoreductase [10,12]. Studies reported herein showed that *B. thailandensis* MSMB43 is not only a new source of FK228 but also a significantly better source of FK228. This bacterial strain produced 67.7 mg/L of FK228 by simple fermentation in shaking flasks; by fed-batch laboratory-scale fermentation *B. thailandensis* MSMB43 achieved the highest titer of 115.9 mg/L, which is 95-fold higher than that of *C. violaceum* No. 968 under the identical fermentation conditions. Moreover, the titer of FK228 production by *B. thailandensis* MSMB43 can be further improved up to 168.5 mg/L in fed-batch fermentation through overexpression of a heterologous regulatory gene *depR*. Therefore, the wild-type *B. thailandensis* MSMB43 or its engineered derivative could potentially be a good starting point for an industrial process to improve FK228 production for its expanding use in therapy.

### 4.2. Evolution of the FK228 biosynthetic gene cluster

Natural product biosynthetic gene clusters are among life's most diverse and rapidly evolving genetic elements [37]. One of the underlying mechanisms for the evolution of many natural product biosynthetic genes and gene clusters is horizontal gene transfer (HGT), often facilitated by mobile elements such as transposons [38,39]. A classic example of HGT is the transmission of NRPS genes responsible for the production of  $\beta$ -lactam antibiotics (e.g., penicillins and cephalosporins), evidently from bacteria to bacteria and from bacteria to fungi [40,41]. A more recent example is the andrimid gene clusters from *Pantoea agglomerans* CU2194 and *Vibrionales bacterium* AWAT-3; the only syntenic difference is the insertion of a single gene at the 3' end of the latter gene cluster [37,42]. The striking similarity between the *dep* gene clusters from *C. violaceum* No. 968 and *B. thailandensis* MSMB43 revealed by this study suggests that the gene cluster most likely have been horizontally transferred from one species to another. Phylogenetically *C. violaceum* No. 968 belongs to *Neisseriales*, while *B. thailandensis* MSMB43 belongs to *Burkholderiales*; both, however, are within the order of betaproteobacteria. The flanking regions of the *Cv\_dep* gene cluster in *C. violaceum* No. 968 does not contain apparent mobile elements but rather house-keeping genes [9], while the flanking regions of *Bth\_dep* gene cluster in *B. thailandensis* MSMB43 contain several putative transposons at each side (data not shown). DNA statistics showed that both *dep* gene clusters have a G+C content of 69–70%, while the G+C content of the flanking regions is about 63% in *C. violaceum* No. 968, or 65% in *B. thailandensis* MSMB43, suggesting that the *dep* gene clusters might have originated from a third organism with a higher G+C genome. Along with the intriguing but unanswered question of evolution, it is equally intriguing yet unanswered about why various bacterial species produce a small molecule FK228 which targets histone deacetylases in eukaryotic epigenome [8].

### Declarations of interest

The authors declare no financial or commercial conflict of interest.

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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.2018.10.011>.

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