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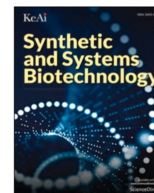

Original Research Article

Novel switchable ECF sigma factor transcription system for improving thaxtomin A production in *Streptomyces*

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ABSTRACT

The application of the valuable natural product thaxtomin A, a potent bioherbicide from the potato scab pathogenic *Streptomyces* strains, has been greatly hindered by the low yields from its native producers. Here, we developed an orthogonal transcription system, leveraging extra-cytoplasmic function (ECF) sigma (σ) factor 17 (ECF17) and its cognate promoter P_{ecf17}, to express the thaxtomin gene cluster and improve the production of thaxtomin A. The minimal P_{ecf17} promoter was determined, and a P_{ecf17} promoter library with a wide range of strengths was constructed. Furthermore, a cumate inducible system was developed for precise temporal control of the ECF17 transcription system in *S. venezuelae* ISP5230. Theoretically, the switchable ECF17 transcription system could reduce the unwanted influences from host and alleviate the burdens introduced by overexpression of heterologous genes. The yield of thaxtomin A was significantly improved to 202.1 ± 15.3 μ g/mL using the switchable ECF17 transcription system for heterologous expression of the thaxtomin gene cluster in *S. venezuelae* ISP5230. Besides, the applicability of this transcription system was also tested in Streptomyces alb*us* J1074, and the titer of thaxtomin A was raised to as high as 239.3 ± 30.6 μg/mL. Therefore, the inducible ECF17 transcription system could serve as a complement of the generally used transcription systems based on strong native constitutive promoters and housekeeping σ factors for the heterologous expression of valuable products in diverse *Streptomyces* hosts.

1. Introduction

Thaxtomin A is a nitrated diketopiperazine bio-herbicide produced by the potato scab pathogens *Streptomyces acidiscabies, Streptomyces scabies*, and *Streptomyces turgidiscabies* [1–3]. Considering its attractive environmental compatibility and rapid degradation in natural environment, the US Environmental Protection Agency has approved thaxtomin A as key active constituent of bio-herbicides for sale for pre-and post-emergence weed control in 2012 [4]. Unfortunately, the low yields in the native producers of thaxtomins have greatly limited their applications in agriculture. Over the past years, efforts have been investigated on heterologous expression of thaxtomin gene cluster in different hosts to achieve overproduction of thaxtomin A and the highest reported yield has climbed to 168 mg/L [5], but still lag far behind the demand of industrialization. The most substantial issue is therefore design and construction of pathways lead to the optimal production of target

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metabolites.Synthetic biology tools enable the reconstruction of biosynthetic gene culsters in a predictable manner. As the first dedicated phase of gene expression, transcription frequently serves as the target for gene regulation. As such, promoter engineering has been widely used for strain improvement and metabolites elicitation. Various constitutive promoters, including *ermE*p [6], SF14p [7], *kasO*p* [8], and their derivatives, have already been used in the successful production of different natural products [9]. These constitutive promoters are generally controlled by housekeeping σ factors, e.g., the transcription initiations of SF14p and *kasO*p* are dependent on HrdB, a σ 70 regulates a large number of housekeeping genes involved in the primary metabolism of *Streptomyces* [10]. Competition with the essential endogenous genes for available σ factors would affect the maximum activities of those promoters [11], which is unfavorable for the high production of valuable products in most cases. Besides housekeeping σ factors, cells encode a number of alternative σ factors responsible for sensing intercellular and intracellular signals to maintain their homeostasis [12,13]. These σ factors are usually under tight control by corresponding *anti*-σ factor [14]. Group 4 alternative σ factors, also known as ECF σ factors, have the simplest structure with only σ4 and σ2 two domains. Upon activation, the two domains bind to the promoter's −35 and −10 regions separately and recruit RNA polymerase core enzyme to initiate transcription [15]. In general, ECF σ factors only regulate their cognate promoters because of the different promoter recognition and unwinding mechanisms [16]and the inefficient promoter melting ability [17]. It was believed that there is little cross-talk between the ECF σ factors and the endogenous housekeeping σ factor [18–21], which makes ECF σ factors and their cognate promoters appropriate orthogonal candidates for engineering genetic switches to reduce the unwanted influences from Streptomyces hosts.

In addition, constitutive high expression of heterologous genes might cause a growth burden to the host cells [22]. To achieve dynamic control of gene expression in *Streptomyces*, inducible promoters were usually employed. Besides the naturally occurring promoters like *tipA*p induced by thiostrepton [23], *gylP* induced by glycerol [24], *nitA*p induced by caprolactam [25], Potr* induced by oxytetracycline [26], and *xylA*p induced by D-xylose [27], there are several artificial inducible promoters constructed by combining constitutive promoters with repressors such as TetR [28], CymR [29] and RolR [29] that respond to anhydrotetracycline, cumate, and resorcinol, respectively. Generally, the artificial inducible promoters like the CymR-based promoters exhibited much better dynamic ranges and stringencies than the naturally occurring ones [30,31].

In this work, we first developed a heterologous transcription system based on the ECF17 σ factor and its cognate promoter P_{ecf17} in *Streptomyces venezuelae* ISP5230. A P_{ecf17} promoter library with a wide range of strengths was then constructed by minimizing the P_{ecf17} promoter and performing saturation mutations of the −35 region to −10 region sequence. The transcriptional activity of the strongest P_{ecf17} promoter mutant is approximately 12-fold higher than that of the constitutive strong *kasO*p* promoter. Further, we employed a cumate inducible system to switch off/on the expression of the ECF17 σ factor to realize temporal control of the ECF17 transcription system. Using the switchable ECF17 transcription system to refactor the thaxtomin gene cluster, the thaxtomin A yield was improved to 202.1 ± 15.3 *μg/mL* in *S. venezuelae* ISP5230 (Fig. 1). We also demonstrated the applicability of this system in another Streptomyces host, S. *albus* J1074, and gained a thaxtomin A yield as high as 239.3 ± 30.6 μg/mL.

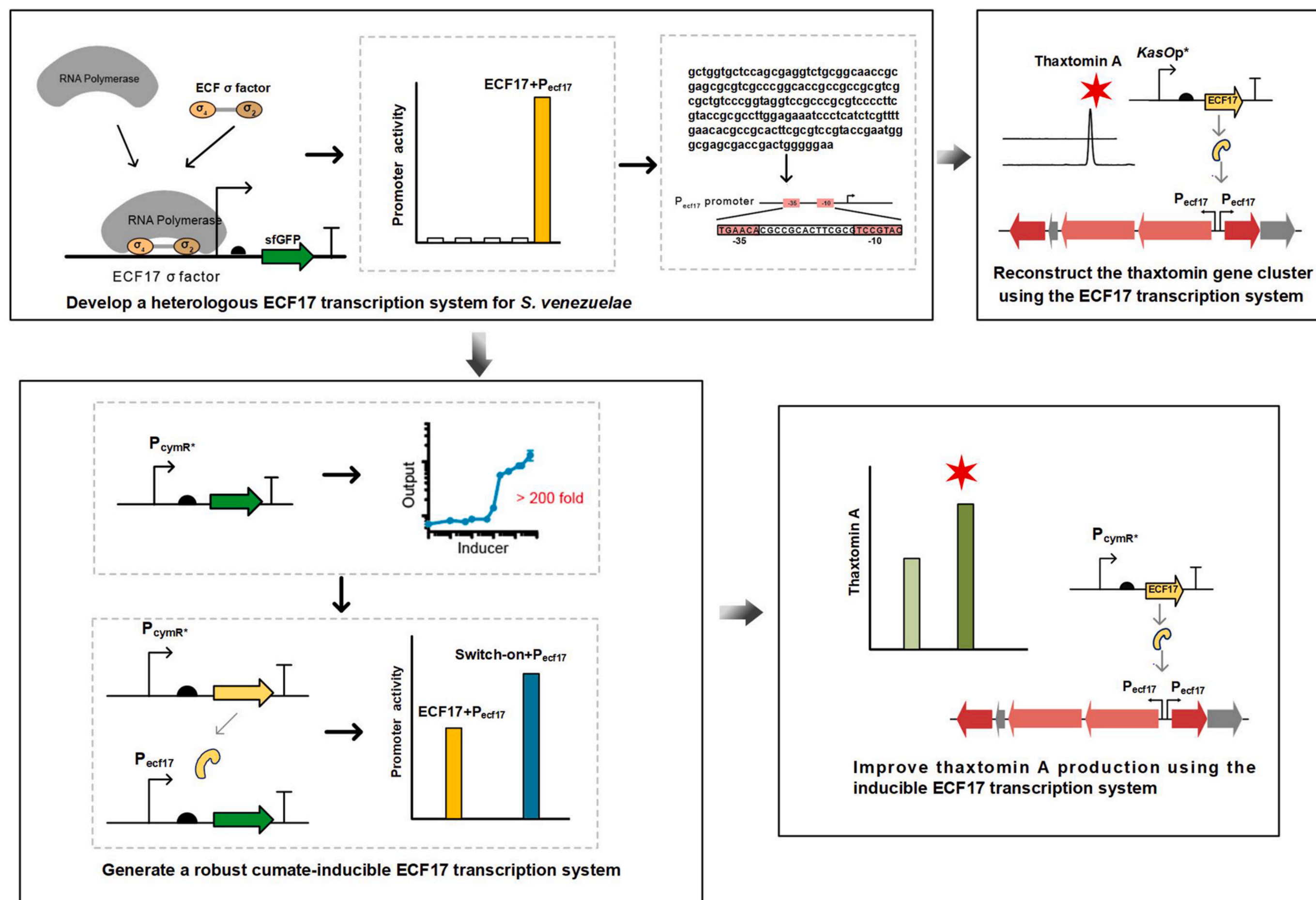

Fig. 1. Overall scheme of the novel switchable ECF17 transcription system for improving thaxtomin A production.

2. Materials and methods

2.1. Strains, media, and reagents

Strains and plasmids used in this study were listed in Table 1. *Escherichia coli* DH10B was used for molecular cloning and plasmid propagation. *E. coli* ET12567/pUZ8002 [32] was used for *E. coli-Streptomyces* conjugation. *E. coli* strains were cultured in LB medium supplemented with appropriate antibiotics (50 mg/L apramycin, 50 mg/L hygromycin, 25 mg/L nalidixic acid). *Streptomyces acidiscabies* ATCC 49003 and *S. albus* J1074 [33] were grown on MS plates (20 g/L soybean flour, 20 g/L mannitol, and 20 g/L agar) for spore preparation. *S. venezuelae* ISP5230 was grown on MYM plates (4.2 g/L D-(+)-maltose monohydrate, 4 g/L yeast extract, 4 g/L malt extract, and 20 g/L agar) for spore preparation. For seed cultivation, *S. venezuelae* was grown in liquid MYM medium, while *S. acidiscabies* and *S. albus* were grown in TSB medium (30 g/L tryptic soy broth (BD)). For fermentation cultivation, *S. venezuelae* and *S. acidiscabies* were grown in oat bran broth (OBB) medium (20 g/L Oat, pH 7.2), and *S. albus* was grown in 0BB or B medium (5 g/L soluble starch, 2 g/L tryptone, 2 g/L yeast extract, 10 g/L soybeans, 4 g/L NaCl, 0.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, and 2 g/L $CaCO_3$). Phosphate buffered saline (PBS) buffer (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, and 0.24 g/L KH_2PO_4) and P10 buffer (sucrose 10.3%, K_2SO_4 1.43 mM, $MgCl_2$ 9.9 mM, KH_2PO_4 0.367 mM, $CaCl_2$ 25 mM, 2-(*N*-Morpholino) ethanesulfonic acid, MES 10 mM) were used for protoplast preparation.

2.2. Construction of the basic clone vectors

We built three shuttle vectors for the conjugation from *E. coli* into the *S. venezulae* and *S. albus* containing the cognate *attB* site for the convenience of molecular cloning. Each shuttle vector contains four separate modules, including *E. coli* origin of replication, a selectable antibiotic resistance cassette, the integrase and its corresponding *attP* site, RP4 origin of transfer (*oriT*) [34]. Further, each shuttle vector was designed to contain the *Bsa*I restriction site for Golden gate assembly [35]. Specifically, the pTHS vector contains pSC101 origin of replication, hygromycin resistance, and TG1 integration system (TG1 integrase-$attP^{TG1}$) [36]; the pPAP-PT vector contains P15A origin of replication, apramycin resistance, and φC31 integration system ($\varphi C31$ *integrase* $-attP^{\varphi C31}$) [37], a ribozyme RiboJ [36], a RBS (BBa_B0034 (RBS, http://parts.igem.org/Part:BBa_B0034)) and the *sfgfp* reporter gene (Fig. S1A).

2.3. Functional analysis of ECF σ factor/P_{ecf} pairs in Streptomyces

We used the pTHS plasmid to construct the ECF_x plasmid. Specifically, the *kasO*p* promoter, rbs-100 ribosome binding site (RBS), codon-optimized ECF *σgenes* (*Table S*1), *and two flanking Bsa*I restriction sites were synthesized by the GenScript company, and assembled on the pTHS plasmid via Golden gate method to generate eight pTHS -ECFx plasmids (Fig. S1B). P_{ecf} promoters (Table S1) synthesized by GenScript company were assembled on the pPAP-PT plasmid via Golden gate assembly to generate 8 pPAP-P_{ecfx} plasmids. The *kasO*p* promoter was inserted directly in front of the RiboJ-BBa_B0034-*sfgfp* cassette of pPAP-PT to generate the pPAP-*kasO*p* plasmid as a positive control.

2.4. Construction of the P_{ecf17} promoter library

To identify the minimal P_{ecf17} promoter, the truncated versions of P_{ecf17} promoter were ordered from GenScript company, and inserted to the pPAP-PT plasmid via Golden gate assembly to generate varied pPAP-P_{ecf17}-No. plasmids (Fig. S1C), here the No.130 bp, 105 bp, 85 bp, 65 bp, 55 bp, 45 bp, 35 bp, 25 bp, 20 bp, 15 bp, 10 bp, 5 bp, and 0 bp denote the DNA lengths upstream of the −35 region (TGAAC) (Table S1). After the minimal P_{ecf17} promoter was determined, a P_{ecf17} promoter library was

Table 1
Strains and plasmids used in this study.

Strain or Plasmid	Relevant genotype	Reference or source
E.coli DH10B	Host for molecular cloning and measurement of P_{ecf} promoter activity	Thermo Scientific
E. coli ET12567/pUZ8002	Donor strain for conjugation between *E.coli* and *Streptomyces*	[32]
S. acidiscabies ATCC 49003	Native thaxtomin A producer	CGMCC 4.1789
S. venezuelae ISP5230	Wild type, *Streptomyces* host strain for heterologous expression	ATCC 10712
S.v/thax	pPAS-thax integrated *S. venezuelae* ISP5230	This study
S.v/thax-P_{EA}	pPAS-thax-P_{EA} integrated *S. venezuelae* ISP5230	This study
S.v/ECF17	pTHS-*kasOp**-ECF17 integrated *S. venezuelae* ISP5230	This study
S.v/thax-P_{ecf17}	pPAS-thax-P_{ecf17} integrated *S. venezuelae* ISP5230	This study
S.v/ECF17::thax-P_{ecf17}	pPAS-thax-P_{ecf17} and pTHS-*kasOp**-ECF17 integrated *S. venezuelae* ISP5230	This study
S.v/ECF17-Switch	pTHS-CymR*-Switch integrated *S. venezuelae* ISP5230	This study
S.v/ECF17-Switch::thax-P_{ecf17}	pPAS-thax-P_{ecf17} and pTHS-CymR*-Switch integrated *S. venezuelae* ISP5230	This study
S. albus J1074	Wild type, *Streptomyces* host strain for heterologous expression	[33]
S.a/thax	pPAS-thax integrated *S. albus* J1074	This study
S.a/thax-P_{EA}	pPAS-thax-P_{EA} integrated *S. albus* J1074	This study
S.a/ECF17	pTHS-*kasOp**-ECF17 integrated *S. albus* J1074	This study
S.a/thax-P_{ecf17}	pPAS-thax-P_{ecf17} integrated *S. albus* J1074	This study
S.a/ECF17::thax-P_{ecf17}	pPAS-thax-P_{ecf17} and pTHS-*kasOp**-ECF17 integrated *S. albus* J1074	This study
S.a/ECF17-Switch	pTHS-CymR*-Switch integrated *S. albus* J1074	This study
S.a/ECF17-Switch::thax-P_{ecf17}	pPAS-thax-P_{ecf17} and pTHS-CymR*-Switch integrated *S. albus* J1074	This study
pPAP	shuttle vector, containg *E.coli* origin of replication, P15A, apramycin resistance, φ C31 integration system	This study
pPAP-PT	pPAP harboring *sfgfp* reporter gene	This study
pPAP-P_{ecfx}	pPAP-PT harboring P_{ecfx} promoter controlling the transcription initiation of *sfgfp* reporter gene (**P_{ecfx}:** $Pecf_{11_3726}$, P_{ecf11_987}, P_{ecf16_3622}, P_{ecf16_973}, P_{ecf34_1384}, P_{ecf27_4265}, P_{ecf38_1322}, P_{ecf17_1458})	This study
pPAP-P_{ecf17}	pPAP-PT harboring P_{ecf17} promoter controlling the transcription initiation of *sfgfp* reporter gene	This study
pPAP-$P_{ecf\text{-}No.}$	pPAP-P_{ecf} with truncated P_{ecf17} promoter (**$P_{ecf\text{-}No.}$:** 130 bp, 105 bp, 85 bp, 65 bp, 55 bp, 45 bp, 35 bp, 25 bp, 20 bp, 15 bp, 10 bp, 5 bp, 0 bp)	This study
pPAP-$P_{ecf17\text{-}M}$	pPAP-P_{ecf17} minimal promoter, and the mutated promoter sequence to construct promoter library	This study
pPAP-*kasOp**	pPAP-PT harboring *kasOp** promoter for the transcription of *sfgfp* reporter gene, positive control	This study
pTHS	shuttle vector, containg *E.coli* origin of replication, pSC101, hygromycin resistance, TG1 integration system	This study
pTHS-*kasOp**-ECFx	pTHS harboring ECF sigma factor (ECF11_3726, ECF11_987, ECF16_3622, ECF16_973, ECF34_1384, ECF27_4265, ECF38_1322, ECF17_1458)	This study
pTHS-*kasOp**-ECF17	pTHS harboring ECF17 σ factor	This study
pTHS-CymR	pTHS harboring CymR repressor, and a cumate-inducible hybrid P_{CymR*} promoter controlling *sfgfp* expression	This study
pTHS-CymR*	pTHS harboring CymR* (CymR-CI434) repressor, and a cumate-inducible hybrid	This study

(continued on next page)

Table 1 (*continued*)

Strain or Plasmid	Relevant genotype	Reference or source
	P_{CymR*} promoter controlling *sfgfp* expression	
pTHS-CymR*-Switchx	pTHS-CymR* with sfGFP reporter replaced by ECF17 σ factor with different RBSs (SR3: pTHS-CymR*-Switch 1; SR15: pTHS-CymR*-Switch 2; SR33:pTHS-CymR*-Switch 3, SR41: pTHS-CymR*-Switch 4)	This study
pTHS-CymR*-Switch	pTHS-CymR* with SR33-ECF17 σ factor under the control of cumate-inducible P_{CymR*} promoter	This study
pPAS	shuttle vector, containg *E.coli* origin of replication, pSC101, apramycin resistance, φ C31 integration system	This study
pPAS-thax	pPAS harboring thaxtomin gene cluster	This study
pPAS-thax-P_{EA}	pPAS harboring bidirectional P_{EA} promoter refactored thaxtomin gene cluster	This study
pPAS-thax-P_{ecf17}	pPAS harboring bidirectional P_{ecf17} promoter refactored thaxtomin gene cluster	This study

constructered by saturation mutagenesis. Specifically, the −35 box was divided into two parts (5' -TGA and 5′-ACA); the −10 box was also divided into two parts (5′-CGT and 5′-AC); and the 15 bp spacer sequence between −35 box and −10 box was divided into five parts (5′-CG, 5′-CC, 5′-GCAC, 5′-TTCG, and 5′-GT) (Fig. S1D). A P_{ecf17} promoter library was then constructered by synthesizing degenerate primers (S1–F/R, S2–F/R, S3–F/R, S4–F/R, S5–F/R, S6–F/R, S7–F/R, S8–F/R, S9–F/R, respectively) (Table S2) containing 5′-NN(N)(N) to replace any of the regions mentioned above, and using overlap extension PCR to obtain the complete promoter sequences, which were then assembled on the pPAP-PT plasmid via Golden gate to generate a series of pPAP-P_{ecf17}-M plasmids with different P_{ecf17} promoters (Fig. S1D).

2.5. Construction of the switchable ECF17 transcription system

The cumate inducible system containing a hybrid P_{CymR*} promoter and a CymR* repressor was first constructed to realize temporal control in *Streptomyces*. The hybrid cumate-inducible P_{CymR*} promoter was obtained by inserting two CymR operator binding sites (CuO) into the up- and down-stream of the SP43 constitutive promoter (Table S1, Fig. S2). The CymR* repressor was generated by fusing the CI434 oligomerization domain to the C-terminus of the CymR repressor as described [38], which was controlled by the SP08 promoter [39](Table S1). The P_{CymR*} promoter, and the CymR* expression cassette together with RiboJ-B-Ba_B0034-*sfgfp* were inserted into the pTHS plasmid via Gibson assembly to generate pTHS-CymR*. As a control, we replaced the CymR* repressor with CymR to generate the pTHS-CymR plasmid.

The cumate-inducible system was then used to control the expression of the ECF17 σ factor. We tested 4 different RBSs (SR3, SR15, SR33, and SR41) [39] (Table S1) to fine-tune the translation initiation rate [40] and the expression level of ECF17 σ factor, and the RigoJ-BBa_B0034-*sfgfp* sequence was replaced with the RBS-ECF17 sequence via Gibson assembly to generate pTHS-CymR*-switch-x. (SR3:pTHS-CymR*-Switch 1; SR15:pTHS-CymR*-Switch 2; SR33: pTHS-CymR*-Switch 3, SR41: pTHS-CymR*-Switch 4).

2.6. Reconstruction of the thaxtomin gene cluster with P_{EA} or P_{ecf17} promoter

Two CRISPR/Cas9 target protospacer sequences were selected from *txtE* and *txtA* promoter regions in the thaxtomin A cluster. The DNA templates of sgRNA-txtE-F and sgRNA-txtA-R were generated via overlap extension of sgRNA-txtE/sgRNA-txtA, guide RNA-F plus guide RNA-R (Table S2), and sgRNA-txtE-F and sgRNA-txtA-R were transcribed *in vitro* with HiScribe™ T7 Quick High Yield RNA Synthesis Kit(NEB). Then, sgRNAs were purified using the RNApure Rapid RNA Kit (BioMed). The Cas9 expression and purification were performed following established protocols [41]. The exchanged bidirectional SP42-SP43 promoter P_{EA} or the bidirectional P_{ecf17} promoter (combination of wild type P_{ecf17} with the minimal P_{ecf17} promoter) containing two ~30 bp overlaps with the corresponding ends of the thaxtomin gene cluster were ordered from GenScript company (Table S1). The pPAS-thax plasmid [41] harboing the thaxtomin gene cluster from *S. acidiscabies* ATCC 49003 (~10 μg) was digested with Cas9 guided by sgRNA-txtE-F and sgRNA-txtA-R at 37 °C for 2 h (Fig. S3). The digested DNA was then ethanol precipitated and resuspended in 20 μL DNase-free water. The exchanged bidirectional SP42-SP43 promoter P_{EA} or the bidirectional P_{ecf17} promoter (combination of wild type P_{ecf17} with the minimal P_{ecf17} promoter) (Table S1) containing two ~30 bp overlaps with the corresponding ends of the thaxtomin gene cluster was ligated separately with the recovered pPAS-thax fragments by Gibson assembly to generate the pPAS-thax-P_{EA} and pPAS-thax-P_{ecf17} (Fig. S3). The correct clones were verified by colony PCR with p-PF and p-PR primers (Table S1).

2.7. Construction of the thaxtomin gene cluster expressing strains

The pPAS-thax plasmid was transferred into *E. coli* ET12567/pUZ8002, and then conjugated into *S. venezuelae* ISP5230 and *S. albus* J1074 to generate *S.v*/thax and *S.a*/thax, repsectively. The pPAS-thax-P_{EA} plasmid was introduced into *S. venezuelae* ISP5230 and *S. albus* J1074 by the same way to generate *S.v*/thax-P_{EA} and *S.a*/thax-P_{EA}, repsectively. *S.v*/ECF17 and *S.a*/ECF17 were constructed using the pTHS-*kasOp**-ECF17 plasmid smilarly. The pPAS-thax-P_{ecf17} plasmid was conjugated into *S.v*/ECF and *S.a*/ECF to generate *S.v*/ECF17:thax-P_{ecf17} and *S.a*/ECF17:thax-P_{ecf17}, repsectively. *S.v*/ECF17-Switch and *S. a*/ECF17-Switch were constructed by introducing the pTHS-CymR*-Switch plasmid into *S. venezuelae* ISP5230 and *S. albus* J1074, repsectively. The pPAS-thax-P_{ecf17} plasmid was then conjugated into *S.v*/ECF17-Switch and *S.a*/ECF17-Switch to generate *S.v*/ECF17-Switch: thax-P_{ecf17} and *S.a*/ECF17:thax-P_{ecf17}, repsectively.

2.8. Determination of cell growth and sfGFP expression using the multimode Varioskan LUX microplate reader

Spore suspensions of *S. venezuelae* strains were inoculated into 15 mL liquid MYM and incubated for 48 h as a seed culture, and 200 μL of seed cultures were transferred into 2 mL of MYM medium in 24 deep plate. Samples were taken and detected at different time points. The biomass was measured by OD_{600}, and promoter activitie was monitored by Tecan Infinite 200 Pro plate reader (Thermo Scientific) with excitation at 485/9 nm and emission at 515/20 nm. The experiments were performed with three biological replicates.

2.9. Thaxtomin A production and HPLC analysis

S. venezuelae were cultured in MYM and *S. albus* strains were cultured in TSB medium at 30 °C for 48 h, and 2 mL of the resultant seed cultures were used to inoculate 50 mL of OBB or B medium in 250 mL flasks. The cultures were incubated at 30 °C, 250 rpm for 5 days. For thaxtomin A detection, 1 mL of fermentation broth was extracted with 10 mL of methanol, and the mycelium was removed by centrifugation at 8,000 rpm for 10 min. The supernatants were filtered through a 0.22 μm pore-size nylon membrane (JIN TENG) and subjected to an Agilent 1100 HPLC system (C18 column: 5 μm × 250 mm × 4.6 mm (Agilent); Mobile phase: acetonitrile: water (40:60), 1 mL/min; Detected at 380 nm). We purchased thaxtomin A standard from Sigma (catalog: SML1456) and established a calibration curve, the concentration of thaxtomin A was calculated based on their corresponding peak area under HPLC trace.

2.10. Confocal laser-scanning fluorescence microscopy analysis of Streptomyces

Streptomyces strains were grown in 24 deep-well plates filled with 2 mL of appropriate liquid medium at 30 °C for 24–48 h. Mycelia from 1 mL liquid cultures were collected by centrifugation at 8,000 rpm for 10 min. The pellets were washed twice with PBS buffer, and resuspended in the same buffer. Samples were examined under a leicaSP8 laser-scanning microscope (Leica) at an excitation wavelength of 488 nm and emission wavelengths of 500–550 nm.

2.11. Quantitation of sfGFP expression by flow cytometry

Quantitation of sfGFP expression in *E. coli* DH10B was performed as described [38]. A total of 50,000 events were recorded. Quantitation of sfGFP expression in *Streptomyces* was performed using a flow-cytometry-based method [39]. Broth culture (1 mL) containing the mycelia was collected by centrifugation at 8,000 rpm for 10 min, washed by 10% (wt/vol) sucrose once, resuspended in 1 mL of the P10 buffer supplemented with 5 mg/mL of lysozyme, and incubated at 37 °C for 1 h to release protoplasts. The protoplasts were analyzed using a FACS Calibur Flow Cytometer (BD Company, USA) with a 488 nm excitation laser and an FL1 (530/30 nm bandpass filter) detector. A total of 50,000 events were recorded. The data were processed using BD FACSuite software and analyzed with FlowJo 9.3.2 software (TreeStar, Inc.). The fluorescence of each sample was recorded as the geometric mean of all of the measured cells and was normalized to the corresponding FSC value, to warrant that only cell size particles were analyzed.

2.12. Statistical analysis

To measure the P_{ecfx} promoter activities and to identify the minimal P_{ecf17} promoter, we used ImageJ to process the fluorescence picture as follows: (*i*) select the whole fluorescent area and measure the “Integrated intensity”; (*ii*) select a region that has no fluorescence as background intensity. Relative promoter activity = [integrated intensity–(selected area × mean fluorescence of background)] ÷ selected area.

To identify the sensitivities of P_{ecf17} promoter to sequence variations, we used one-way analysis of variance (ANOVA) for each mutated region. CV, coefficient of variation; FV, relative (-fold) variation determined by the ration of maximal promoter activity to the minimal promoter activity.Data were analyzed using GraphPad Prism 9 and presented as mean ± standard deviation.

3. Results

3.1. Development of a heterologous ECF transcription system for S. venezuelae

From the 20 well-characterized ECF σ factor/promoter pairs [18], we selected four pairs with high transcriptional activities (ECF11_987 from Vibrio parahaemolyticus RIMD 2210633, ECF11_3276 from *Pseudomonas syringae* pv. tomato str. DC3000, ECF16_973 from Pseudomonas putida KT2440, and ECF16_3622 from Pseudomonas entomophila L48). In addition, we chose four σ factors (ECF17_1458, ECF27_4256, ECF34_1384, and ECF38_1322) from S. coelicolor A3(2), which is taxonomically closer to the host strain *S. venezuelae* ISP5230. The σ factors and their corresponding promoters were cloned into two plasmid backbones, pTHS and pPAP-PT (with an sfGFP encoding gene downstream the promoter), respectively,via one-step Golden gate assembly to generate pTHS-*kasO*p*-ECFx and pPAP-P_{ecfx} (Table 1, Fig. S1B). Each pTHS-*kasO*p*-ECFx and its cognate pPAP-P_{ecfx} plasmid pair was introduced into *S. venezuelae* ISP5230, and the expression levels of sfGFP were quantified using confocal fluorescent microscopy. To exclude potential interfere of the ECF promoters by the host transcription machinery, we introduced the pPAP-P_{ecfx} plasmid to S. venezuelae ISP5230 solely as a negative control. The *sfgfp* gene cloned downstream of the *kasO*p* promoter was employed as a positive control (Fig. 2A).

As shown in Figs. 2B and S4A, among the eight tested ECF σ factor/promoter pairs, only two of them from *S. coelicolor* A3(2) were active in *S. venezuelae* ISP5230. The ECF σ factor/promoter pair ECF17/P_{ecf17} exhibited a strong activity, and the ECF38/P_{ecf38} pair showed relatively weak activity. While, in the negative control strain without the plasmid expressing ECF17 σ factor, the cognate promoter P_{ecf17} was silent, indicating that this promoter could not be activated by the σ factors from *S. venezuelae* ISP5230 in these conditions (Fig. S4A). Flow cytometry was then used to quantify the activity of ECF17/P_{ecf17} precisely in *S. venezuelae*, which showed that the expression level of sfGFP reporter under the control fo ECF17/P_{ecf17} was approximately 5-fold higher than that of kasOp*, a frequently used strong promoter in *Streptomyces* (Fig. 2C).

To facilitate the subsequent modifications of P_{ecf17} promoter, we delineated the minimal promoter sequence, which usually contains the up −35 element, −35 box, −10 box, and a possible transcription starting site. It could also eliminate the unnecessary DNA regions that might affect the promoter activity [42] and alleviate the homologous recombination problem caused by using this promoter sequence repetitively [43,44]. By a serial truncation experiment upstream of the proposed −35 region (TGAAC), we found that when the truncation process reached 20 bp upstream of the −35 region, the fluorescence intensity decreased significantly. Thus, the minimal P_{ecf17} promoter was narrowed to a DNA sequence with a total length of 80 bp (from 25 bp upstream of the −35 region to 28 bp downstream of the −10 region) (Fig. 3A, Fig. S4B).

A P_{ecf17} promoter library was then constructed based on the 80 bp minimal promoter sequence to meet the demand of fine-tune gene expression [45]. We split the sequence from −35 region to −10 region into nine sections and performed saturation mutation for each section to obtain a series of candidates with different strengths. Since the 2 bp sequence upstream of the −10 region is sensitive to mutation, we incorporated them into the −10 region. The critical functional parts of the P_{ecf17} promoter were determined to be TGAACA $(N)_{14}$TCCGTAC (Fig. 3B). Ten different promoters from the mutation libraries were chosen for precise quantification in *E. coli* DH10B and S. venezuelae ISP5230 using flow cytometry.These promoter mutants displayed a wide range of strength and had rank-order consistency in both hosts (Fig. 3C, Table S3), indicating that they have good modularity and portability. In S. venezuelae ISP5230, the lowest promoter activity of the minimal P_{ecf17} mutant (M10) was approximately 1% that of wild-type P_{ecf17}; while the highest one (M1) was raised to about 2.5 fold of that of wild-type P_{ecf17}, which was 12 fold higher than that of kasOp*.

3.2. Production of thaxtomin A in S. venezuelae ISP5230

The ECF17 transcription system was then applied to reconstruct the thaxtomin gene cluster in *S. venezuelae* ISP5230 (Fig. 4A). The native promoters of *txtA* and *txtE* were replaced with P_{ecf17} bidirectional promoter cassette or the strong constitutive promoter cassette SP43-SP42 as a positive control, and the pathway-specific transcriptional activator gene *txtR* was excluded from the gene cluster, yielding the pPAS-thax-P_{ecf17} and pPAS-thax-P_{ecf17} (Fig. 4A, Fig. S3). Introduction of pPAS-thax-P_{EA} into *S. venezuelae* ISP5230 obtained *S.v*/thax-P_{EA}, which gained the capacity to synthesize thaxtomin A (48 ± 9.3 μg/mL) (Fig. 4B), excluding the possibility that the cloned thaxtomin gene cluster was malfunctional. pPAS-thax-P_{ecf17} was introduced into *S. venezuelae* ISP5230 and *S. venezuelae*/ECF17 harboring the plasmid for ECF17 σ factor expression to generate *S.v*/thax-P_{ecf17} and *S.v*/ECF17:thax-P_{ecf17}, respectively. As anticipated, *S.v*/thax-P_{ecf17} without ECF17 σ factor could not produce thaxtomin A; *S.v*/ECF17:thax-P_{ecf17} could synthesize thaxtomin efficiently with the thaxtomin A titer reaching 71.5 ± 5.0 μg/mL, about 50% higher than that in *S.v*/thax-P_{EA} (Fig. 4B).

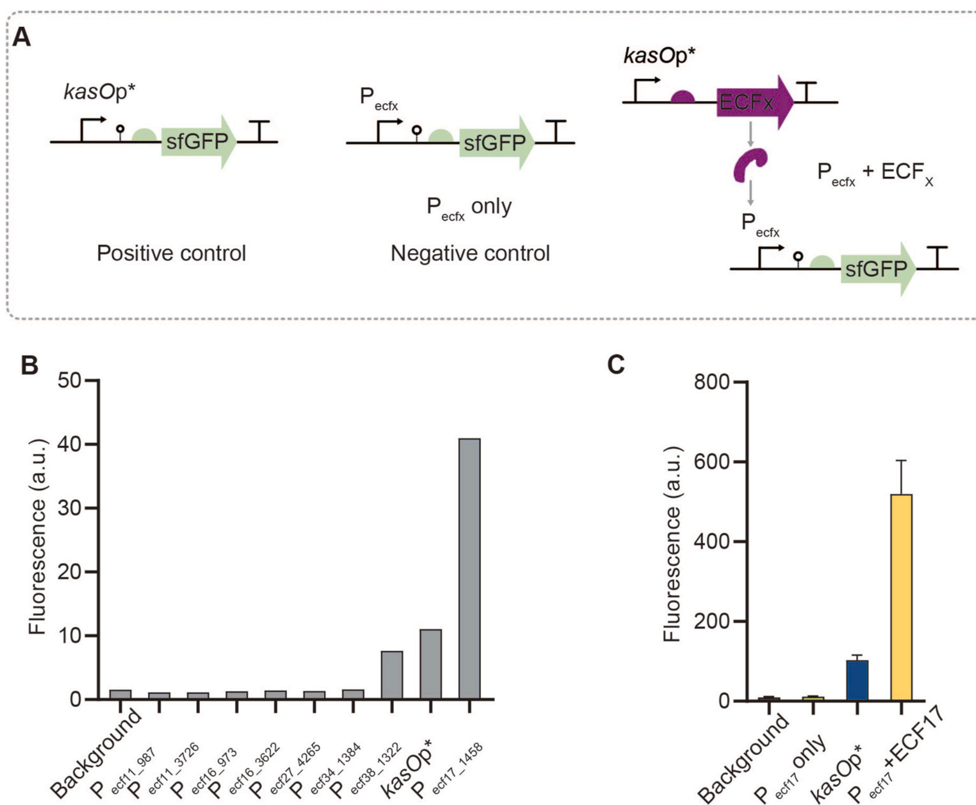

Fig. 2. The functionality of the heterologous ECF transcriptional system in *S. venezuelae* ISP5230. **A**, Genetic layout of the ECF transcription system. (ECF_X: ECF11_3726, ECF11_987, ECF16_3622, ECF16_973, ECF34_1384, ECF27_4265, ECF38_1322 (named as ECF38), ECF17_1458 (named as ECF17); the corresponding P_{ecfx}: P_{ecf11_3726}, P_{ecf11_987}, P_{ecf16_3622}, P_{ecf16_973}, P_{ecf34_1384}, P_{ecf27_4265}, P_{ecf38_1322} (named as P_{ecf38}), P_{ecf17_1458} (named as P_{ecf17}). The *kasO*p* promoter was used as a positive control. The pPAP-P_{ecfx} plasmid in *S. venezuelae* ISP5230 solely was used as a negative control to exclude potential interference from the host transcription machinery. **B**, The activities of ECF σ factors in *S. venezuelae* ISP5230 were monitored by confocal fluorescence microscopy and processed by ImageJ software. **C**, Precise quantification of P_{ecf17} promoter activity using flow cytometry. Data represent the mean ± SD of at least three replicate experiments, a.u. means arbitrary unit, ** indicates significant difference.

3.3. Improving the production of thaxtomin A using the cumate-inducible ECF17 transcription system

High biosynthesis of heterologous genes in the primary growth impose heavy burdens to the hosets. Besides, constitutive expression of ECF17 σ factor is probably toxic to the hosts because of its competition with the housekeeping σ factors for core RNA polymerase along with the growth. So, introduction of an inducible system could alleviate the problems and realize temporal control of gene expression. It has been demonstrated that the engineered CymR* (fusing the oligomerization domain of CI434 to CymR) [38] is able to work cooperatively to generate a sigmoidal response curve in *E. coli* and is appropriate for use as a rigorous genetic switch. A genetic switch based on CymR* was therefore constructed to control the ECF transcription system in *S. venezuelae* ISP5230. The CI434 oligomerization domain was fused to the C terminus of CymR to generate CymR*. A hybrid cumate inducible promoter (P_{CymR*}) was obtained by inserting two cumate operators (CuO) into the up- and down-stream of SP43 promoter (Fig. 5A, Fig. S2). The CymR*-P_{CymR*} induction system was then tested in *S. venezuelae* ISP5230 together with the wild-type CymR system as a control. As shown in Fig. 5B, the CymR* inducible system displayed a much wider dynamic range (>200 fold) than that of the wild-type CymR system (~85 fold), indicating it could be used as a rigorous genetic switch. Subsequently, the ECF17 σ factor encoding gene was put under the control of the CymR*-P_{CymR*} induction system to construct a switchable ECF σ factor transcription system in *S. venezuelae* ISP5230 (Fig. 5C). Four different RBSs (SR3, SR15, SR33, SR41) with varied strengths [39]were tested for fine-tuning ECF17 gene expression. The time-course experiment revealed that it was appropriate to add the cumate inducer at 4 h (Fig. S5). After the design-build-test cycle, the version named CymR*-Switch 3 (containing RBS SR33) outperformed the other combinations (Fig. S4C). Thus, CymR*-Switch 3 was used to control the expression of ECF17. The expression levels of sfGFP were quantified with flow cytometry, of which the P_{ecf17} activity had a 60 fold on/off ratio. The P_{ecf17} promoter activity observed with ECF17 σ factor controlled by the CymR*-Switch 3 was much higher than that of the constitutively expressed ECF17 σ factor (Fig. 5D). Meantime, the growth rates of the *S.v*/ECF17+P_{ecf17} and *S.v*/Switch-on + P_{ecf17} were determined. As shown in Fig. S6A, both strains exhibited lower biomass than wild-type strain. But the *S.v*/Switch-on + P_{ecf17} showed higher biomass than *S.v*/ECF17+P_{ecf17}. These results demonstrate that the cumate-inducible ECF transcription system can indeed decrease the toxicity to the host caused by constitutive expression of ECF17 σ factor. The switchable ECF17 transcription system was then used for thaxtomin A production by introducing CymR*-Switch 3 into *S.v*/thax-P_{ecf17} to generate *S.v*/ECF17-Switch:thax-P_{ecf17} (Fig. 6A). As anticipated, the thaxtomin A yield was as high as 202.1 ± 15.3 μg/mL (Fig. 6B), representing an almost 4 fold improvement compared to the constitutively expressed ECF17 system. The transcriptional levels of the *thax* genes were investigated by real-time PCR and the result confirmed that all the genes in the refactored cluster were turned on at high levels by the addition of cumate. It confirmed that the cumate inducible ECF17 system indeed can strongly improve the expression of target gene cluster at transcriptional level (Fig. S7). On the other hand, due to the burden of ECF17, the biomass of *S.v*/ECF17:thax-P_{ecf17} was less than the control strain *S.v*/thax-P_{EA}, while the *S.v*/ECF17-Switch:thax-P_{ecf17} strain has close biomass to the control strain (Fig. S6B). It emphasized the benefit of alleviation the burden to improving the production of thaxtomin A. Taken together, the data demonstrate that this new cumate-inducible transcription system is effective for activating and improving the expression of target gene clusters in *Streptomyces*.

3.4. Production of thaxtomin A using the inducible ECF17 transcription system in S. albus J1074

To test the applicability of the ECF17 transcription system in other *Streptomyces* strains, pTHS-ECF17 and its cognate pPAP-P_{ecf17} plasmid were introduced into another frequently used *Streptomyces* host *S. albus*

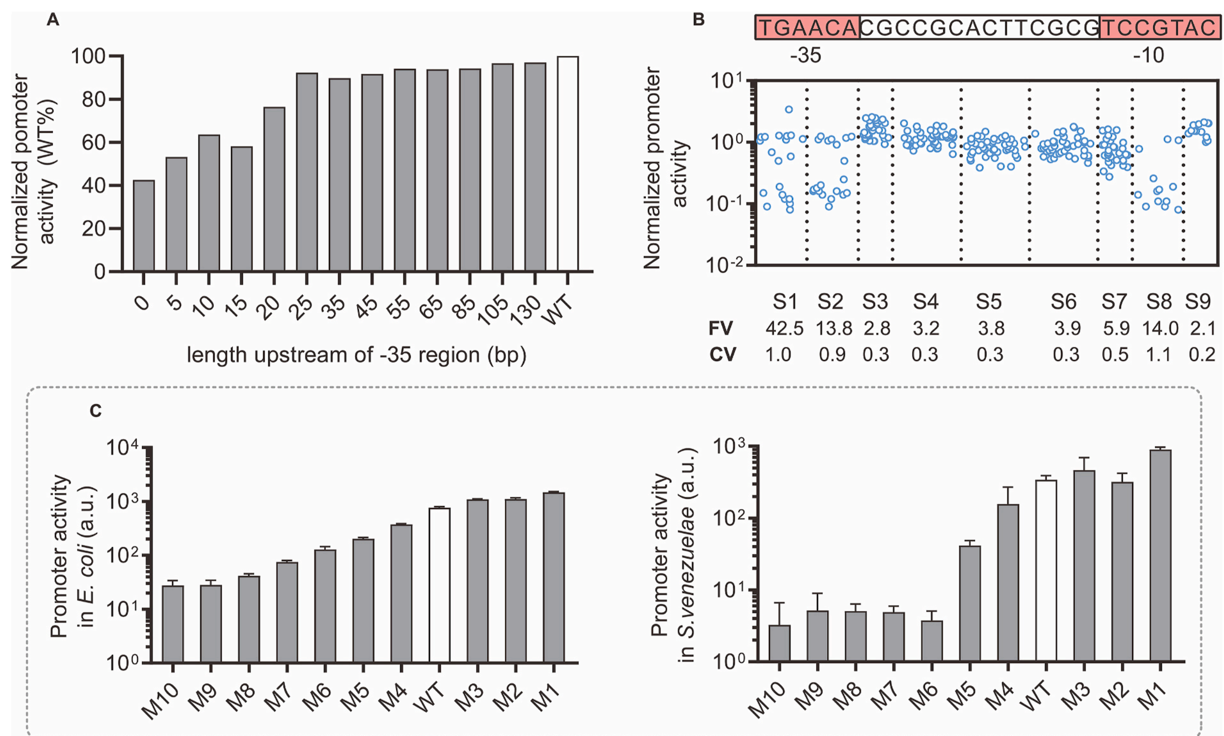

Fig. 3. Identifying and Engineering the minimal P_{ecf17} promoter. **A**, Identification of the minimal P_{ecf17} promoter by serial truncation. Shown here are the relative activities of promoters truncated upstream of the −35 region compared to the wild-type P_{ecf17} promoter, which were monitored by confocal fluorescence microscopy and processed by ImageJ software. **B**, Effects of random mutagenesis on the activity of P_{ecf17}. Each dot represents a randomly selected mutant containing mutations in the corresponding region. FV means fold variation, CV means coefficient of variation, the results indicated that S1, S2, S7, S8 corresponding to −35 and −10 regions are essential for maintaining promoter activity (FV > 5.9, CV > 0.5); S9 is located between the −10 region and transcriptional start site, it is crucial for promoter activity. P_{ecf17} activity is insensitive to the mutation of S3, S4, S5, and S6 regions (FV < 3.9, CV < 0.3). **C**, A P_{ecf17} promoter library generated by random mutation of the −10 and −35 box sequences and their relative activities in *E. coli* and *S. venezuelae* were monitored by Tecan Infinite 200 Pro plate reader with excitation at 485/9 nm and emission at 515/20 nm.
a.u. means arbitrary unit.

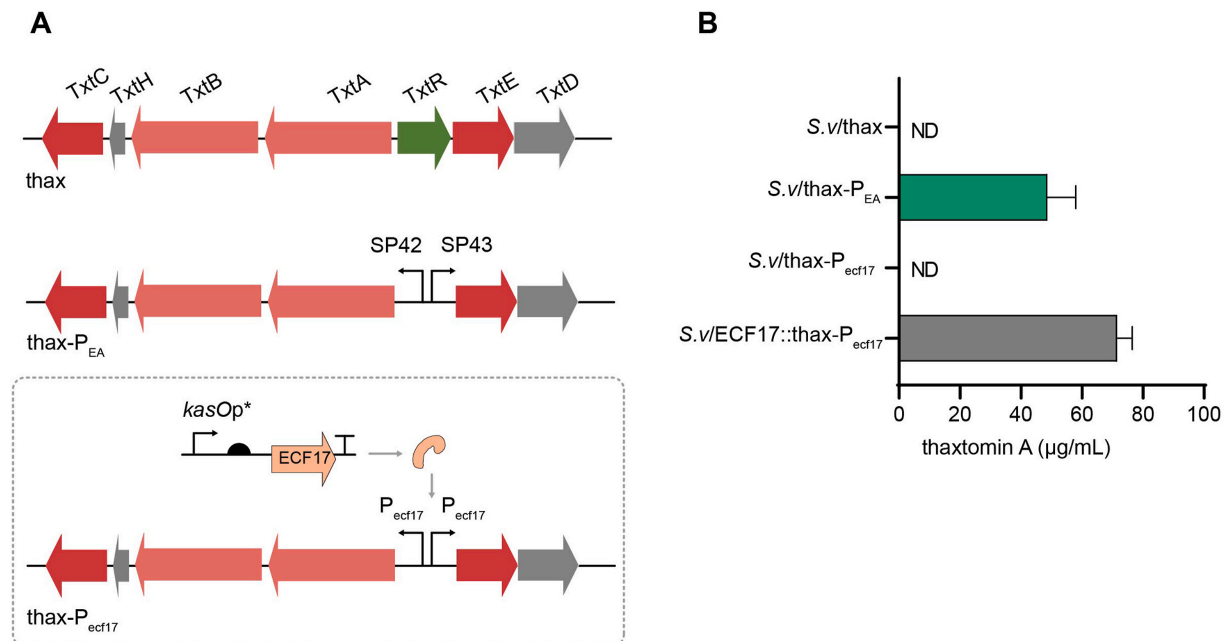

Fig. 4. Reconstruction and heterologous expression of the thaxtomin gene cluster. **A**, Genetic organizations of the thaxtomin gene cluster from *S. acidsabies* ATCC 49003 and the refactored thax-P_{EA} and thax-P_{ecf17} thaxtomin gene clusters (the P_{ecf17} promoter activity depends on the presence of ECF17 σ factor). **B**, Comparison of the thaxtomin A yields in different *S. venezuelae* heterologous expression strains in OBB medium. ND, not detected. Three different exconjugants from each strain were used in the experiment, and the reported values represent the means ± SD from three biological replicates.

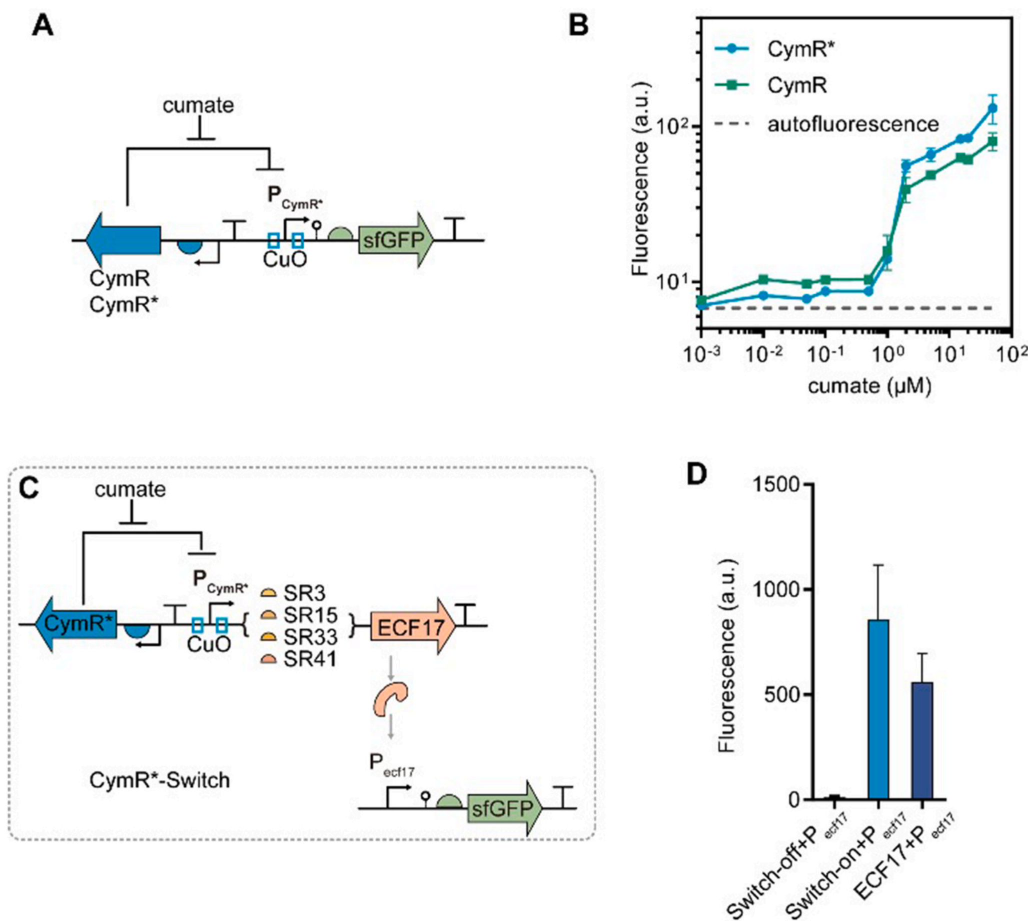

Fig. 5. Design and evaluation of the CymR*-P_{CymR*} switch. **A**, Schematic representation of the cumate-inducible CymR*-P_{CymR*} system. The blue rectangles mean CymR (CymR*) binding site CuO (the same below). Addition of cumate inducer would derepress the P_{CymR*} promoter and lead to the expression of sfGFP reporter. **B**, The corresponding response curves of derepression of CymR or CymR* by adding cumate inducer, which were monitored by flow cytometry. **C**, Fine-tuning the ECF17 expression level with RBSs of different strengths. All the CymR*-Switches were monitored by confocal fluorescence microscopy. **D**, Characterization of the CymR*-Switch 3 (containing SR33) with flow cytometry. The constitutively expressed ECF17 was used for comparison. a.u. means arbitrary unit. The data represent the means ± SD from at least three biological replicates.

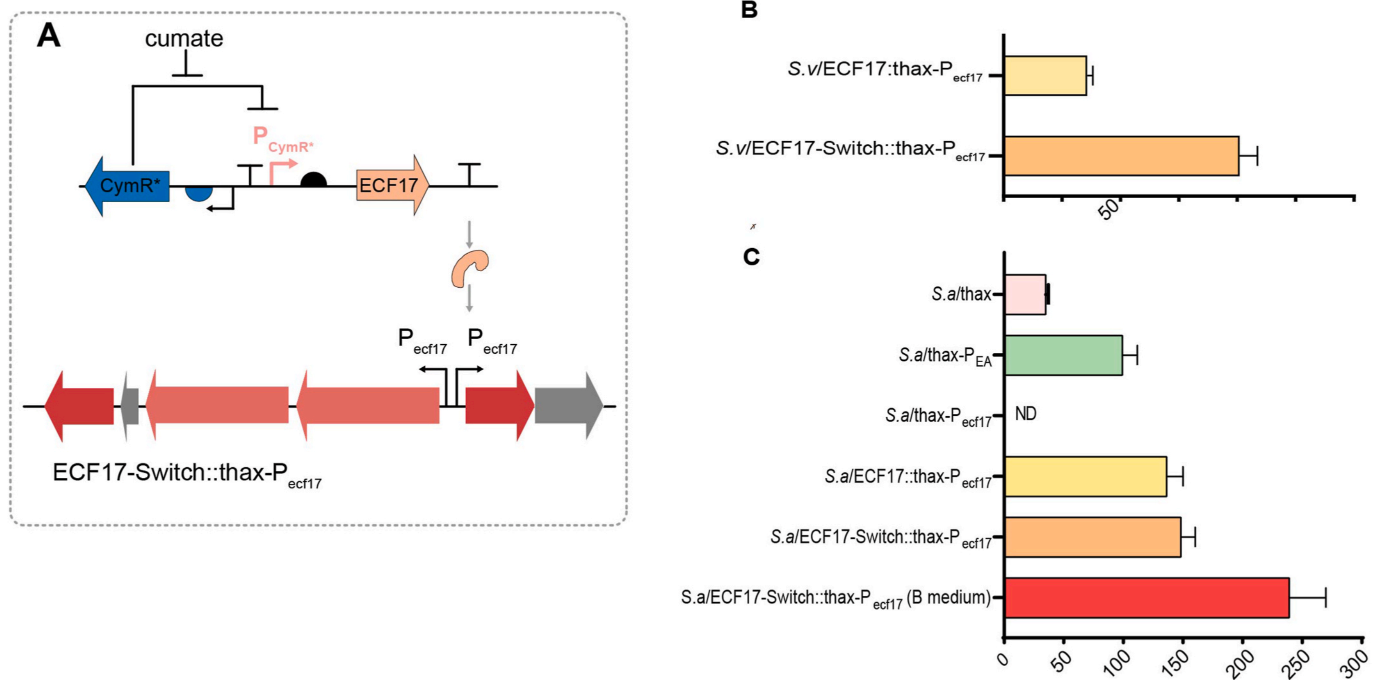

Fig. 6. Improvement of thaxtomin A production using the inducible ECF17 transcription system. **A**, Genetic layout of the engineered gene cluster using the ECF17-Switch system. **B**, Thaxtomin A productions in *S. venezuelae* heterologous expression strains in OBB medium. **C**, Thaxtomin A productions in *S.albus* heterologous expression strains in OBB medium. In addition, we also fermented *S.a/*ECF17-Switch:thax-Pecf17 in B medium. The data represent the means ± SD from three biological replicates.

J1074. The confocal fluorescent microscopy results revealed that this system worked well in *S. albus* J1074 and the P_{ecf17} promoter activity depended on the presence of the ECF17 σ factor (Fig. S4D). The ECF17 transcription system was then used to produce thaxtomin A in *S. albus* J1074. The pPAS-thax-P_{ecf17} plasmid harboring the engineered thaxtomin gene cluster controlled by P_{ecf17} was introduced into *S. albus* J1074, *S. albus*/ECF17 with pTHS-*kasO*p*-ECF17, and *S. albus*/ECF17-Switch with the switchable ECF17 to generate *S.a*/thax-P_{ecf17}, *S.a*/ECF17:thax-P_{ecf17} and *S.a*/ECF17-Switch:thax-P_{ecf17}, respectively. In addition, plasmid pPAS-thax-P_{EA} that harbors the engineered thaxtomin gene cluster controlled by the constitutive promoter pair SP43-SP42 was introduced into *S. albus* J1074 to construct a positive control strain *S.a*/thax-P_{EA}. After 5 days of fermentation in OBB medium, the yield of thaxtomin A in *S.a*/ECF17:thax-P_{ecf17} is 136.5 ±13.4 μg/mL, which was approximately 37% higher than that in *S.a*/thax-P_{EA} (99.3 ± 12.3 μg/mL). For *S.a*/ECF17-Switch:thax-P_{ecf17}, addition of 50 μM cumate switched on the expression of the ECF17 σ factor and led to 148.4± 8.4 μg/mL (Fig. 6C). In our early work, we found that B medium is the optimal fermentation medium for thaxtomin A production in *S.albus* host (data not shown). So, we also fermentated *S.a*/ECF17-Switch:thax-P_{ecf17} in B medium, and the thaxtomin A researched 239.3 ± 30.6 *μg/mL*. It was even higher than that in *S.v*/ECF17-Switch:thax-P_{ecf17} (Fig. 6C). Overall, these results demonstrate that the ECF17 transcription system developed for *S. venezuelae* ISP5230 has a great potential to be applied in the other *Streptomyces* strains.

4. Discussion

The fundamental difficulty in manipulating the biosynthesis of target metabolites arises from the inherent complex metabolic regulation of living cells [46–48]. Moverover, the behavior of synthetic elements strongly depends on the cellular context, which significantly limits their engineering applications. Therefore, many engineered gene circuits may work in unexpected ways or even completely lose their function when placed in different genetic backgrounds [49–51]. To overcome these shortcomings, a wide range of orthogonal and well-characterized elements that have minimal undesired cross-talk with the host cells were developed in *E.coli* [20,51,52]. However, very few of these orthogonal systems were used to *Streptomyces*, a genus that contains some of the most important sources of natural products applied in human medicine, animal health and plant crop protection. In this study, we constructed an orthogonal ECF17 σ factor based transcription system and tested it in two common heteregous hosts *S. venezuelae* ISP5230 and *S. albus* J1074. In both strains, the cognate promoter of ECF17 σ factor, P_{ecf17}, was inactive unless ECF17 σ factor was expressed, indicating that P_{ecf17} could not be recognized by the endogenous σ factors. So such a transcription system will significantly reduce the unwanted influences from the hosts during heterologous expression processes. Furthermore, we designed a novel genetic switch with tight regulation and high activity based on ECF17 transcription activator and cumate-inducible system, which allows fine-tuning of gene expression to desired levels. More importantly, it could be used for driving the expression of large geng cluster. In the case of thaxtomin A, refactoring the thaxtomin gene cluster using the transcription system lead to a dramatic increase in heteregous hosts *S. venezuelae* ISP5230 and *S. albus* J1074.

In conclusion, our work suggests a new excellent regulatory system for engineering secondary metabolites gene clusters, and contributes a step forward toward the industrialization of thaxtomin A bio-herbicide. We expect that the novel cumate-inducible transcription system established in this study will facilitate the production of important natural drugs in *Streptomyces*.

Declaration of competing interest

We have no conflict of interest to declare.

Availability of data and material

Data that supports the finding of this study are available in the main text and the supplementary materials. Ethical approval This article does not contain studies with human participants or animals performed by any of the authors.

Consent for publication

All listed authors have approved the manuscript before submission, including the names and order of authors.

CRediT authorship contribution statement

Xuejin Zhao: Data curation, Formal analysis, Methodology, Writing – original draft. **Weijia Wei:** Data curation, Formal analysis, Methodology, Writing – original draft. **Yeqing Zong:** Data curation, Methodology, Software. **Chaoxian Bai:** Data curation. **Xian Guo:** Data curation. **Hua Zhu:** Supervision, Writing – review & editing. **Chunbo Lou:** Supervision, Project administration.

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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.2022.05.010.

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