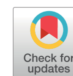
Synthetic and Systems Biotechnology 4 (2019) 40–48

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: http://www.keaipublishing.com/synbio

A LuxR family transcriptional regulator AniF promotes the production of anisomycin and its derivatives in *Streptomyces hygrospinosus* var. beijingensis



Jufang Shen[a,1], Lingxin Kong[a,1], Yan Li[a], Xiaoqing Zheng[a,b], Qing Wang[a], Weinan Yang[a], Zixin Deng[a], Delin You[a,*]

[a] *State Key Laboratory of Microbial Metabolism, Joint International Research Laboratory of Metabolic and Developmental Sciences, School of Life Sciences & Biotechnology, Shanghai Jiao Tong University, Shanghai, China*
[b] *Department of Immunology, Hebei Medical University, Shijiazhuang, Hebei, China*

ARTICLE INFO

Keywords:
Anisomycin
Agricultural Antibiotic120
Biosynthesis
AniF
LuxR regulator
Streptomyces

ABSTRACT

The protein synthesis inhibitor anisomycin features a unique benzylpyrrolidine system and exhibits potent selective activity against pathogenic protozoa and fungi. It is one of the important effective components in Agricultural Antibiotic120, which has been widely used as naturally-originated agents for treatment of crop decay in China. The chemical synthesis of anisomycin has recently been reported, but the complex process with low productivity made the biosynthesis still to be a vital mainstay in efforts. The biosynthetic gene cluster (BGC) of anisomycin in *Streptomyces hygrospinosus* var. beijingensis has been identified in our previous work, while poor understanding of the regulatory mechanism limited the yield enhancement via regulation engineering of *S. hygrospinosus* var. beijingensis. In this study here, we characterized AniF as an indispensable LuxR family transcriptional regulator for the activation of anisomycin biosynthesis. The genetic manipulations of *aniF* and the real-time quantitative PCR (RT-qPCR) revealed that it positively regulated the transcription of the anisomycin BGC. Moreover, the overexpression of *aniF* contributed to the improvement of the production of anisomycin and its derivatives. Dissection of the mechanism underlying the function of AniF revealed that it directly activated the transcription of the genes *aniR-G* involved in anisomycin biosynthesis. Especially, one AniF-binding site in the promoter region of *aniR* was identified by DNase I footprinting assay and an inverted repeat sequence (5′-GGGC-3′) composed of two 4-nt half sites in the protected region was found. Taken together, our systematic study confirmed the positive regulatory role of AniF and might facilitate the future construction of engineering strains with high productivity of anisomycin and its derivatives.

1. Introduction

Streptomyces, the largest genus of *Actinobacteria*, is characterized by a complex secondary metabolism, and produce over two-thirds of the clinically useful natural products that can be used as antibacterial, antifungal, and antitumor drugs [1]. The production of these antibiotics is usually controlled by multiple regulatory proteins that respond to internal physiological and environmental conditions. It has been known that the complex regulatory cascades worked through multiple level regulators, including global regulators, pleiotropic regulators and pathway-specific regulator [2–4]. Transcriptional regulation allows the cell to allocate its valuable resources towards the production of proteins and provides a method for the build-up of desired metabolites [5]. Engineering transcriptional control for the optimization of engineered metabolic pathways requires careful control over the levels and timing of metabolic enzyme expression. However, the utilization of transcriptional control engineering for the construction of high yield strains is largely dependent on the elucidation of the regulatory system.

Anisomycin (Fig. 1a), a pyrrolidine antibiotic, is one of the important effective components in Agricultural Antibiotic120, which has been widely used for treatment of crop decay in China [6], about 13 million acres every year. Owing to the reversible 60S ribosomal subunit binding activity, anisomycin can block peptide bond formation and shows potent selective activity against pathogenic protozoa and fungi, and was commercially available named flagecidin for the treatment of plant pathogenic fungi [7,8]. It has been shown to treat mental

Peer review under responsibility of KeAi Communications Co., Ltd.
* Corresponding author.
E-mail address: dlyou@sjtu.edu.cn (D. You).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.synbio.2018.12.004
Received 14 December 2018; Received in revised form 26 December 2018; Accepted 29 December 2018
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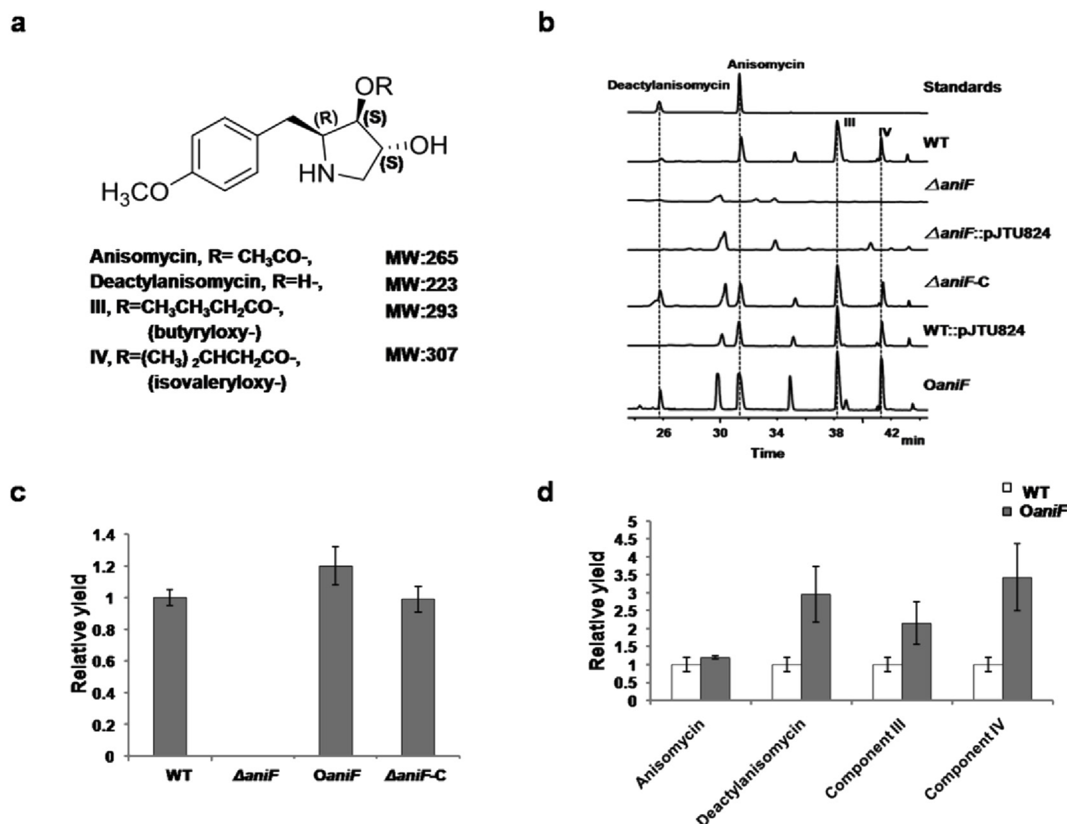

Fig. 1. Effects of deletion and overexpression of *aniF* on the production of anisomycin and its derivatives. (a) The chemical structure of anisomycin and its derivatives. **(b)** The HPLC analysis of the anisomycin and its derivatives productions in WT, Δ*aniF*, Δ*aniF*::pJTU824, Δ*aniF*-C, WT::pJTU824, O*aniF* strains. Anisomycin and deactylanisomycin standards were used as control. **(c)** Quantitative analysis of anisomycin produced in WT, Δ*aniF*, O*aniF* and Δ*aniF*-C strains. **(d)** Comparative analysis of the production of anisomycin and its derivatives in WT and O*aniF*. For comparison, the yield of anisomycin and its three derivatives in the controlled WT are determined as 1 in c and d.

disorders by preventing the synthesis of proteins needed for long-term memory [9]. What's more, anisomycin can suppress Jurkat T cell growth by the cell cycle-regulating proteins so that it can be developed as the potential immunosuppressive drugs [10]. Recently, it has been reported that it can induce cell cycle apoptosis and activate JNKs signal pathway, and thus exhibit potential antitumor activity [10,11]. The diverse biological activities and structural features of anisomycin have attracted the attention of many chemists and biologists. Anisomycin was firstly isolated from *Streptomyces roseochromogenes* and *Streptomyces griseolus* in 1954 and later was also found from *S. hygrospinosus* var. beijingensis [7]. Up to date, three anisomycin analogues (deactylanisomycin, component III and component IV) (Fig. 1a) with different chain length of acyl group have been isolated and been tested for the biological activities. Even with the successful chemical synthesis, the biosynthetic study of anisomycin has remained to be an enigma. Fortunately, the biosynthetic gene cluster (BGC) of anisomycin has been cloned successfully by using a bioactivity-guided library screening approach last year [12]. Nine essential genes (*aniF*, *aniG*, *aniI*, *aniK*, *aniL*, *aniN*, *aniO*, *aniP* and *aniQ*) for the biosynthesis of anisomycin have been identified and the biosynthetic pathway of anisomycin was revealed involving a previously unknown pyrrolidine biosynthesis route [12]. During the process, the genetic deletion of the candidate regulatory gene *aniF* completely abolished the production of anisomycin and other identified biosynthetic intermediates. In view of the wide potential medical application of anisomycin, the detailed characterization of this regulatory gene involved in its biosynthesis is important for practical construction of strains with high anisomycin productivity.

In order to explore the biosynthetic regulatory mechanism and provide insight into future construction of high-yield engineering strains, the regulatory role of AniF was characterized in this study. The genetic complementation of *aniF* restored the production of anisomycin and the overexpression of it contributed to the 1.2-fold improvement of anisomycin productivity. Additionally, in the *aniF*-overexpressing strain the productions of other three derivatives were also increased 2–3 times than that in wild type (WT) strain. Meanwhile, almost all the genes (*aniR-G*) within the BGC were identified to be regulated by AniF. An AniF-binding site was identified within the promoter region of *aniR* by DNase I footprinting and an inverted repeat (5′-GGGC-3′) composed of two 4-nt half sites in the protected region was found. Moreover, for the exploration of the possible regulatory mechanism, the effects of anisomycin and several biosynthetic intermediates on the binding activity of AniF were investigated. Taken together, AniF appeared to activate the expression of the anisomycin BGC and in turn contributed to further accumulation of anisomycin and its derivates. The findings reported here showed that *aniF* could be used for the future construction of high-yield strains.

2. Materials and methods

2.1. Strains, plasmids, and growth conditions

The strains and plasmids used in this study were listed in Table 1. *S. hygrospinosus* var. beijingensis, the WT producer of anisomycin and its derivative strains were grown at 30 °C on solid SFM medium (2% mannitol, 2% soya flour and 1.5% agar) for sporulation. The seed liquid TSBY medium [12] (per liter contained 3% tryptic soy broth, 1% yeast extract, 10.3% sucrose, pH 7.2) and fermentation liquid medium (per liter contained 1% corn starch, 2% soluble starch, 1% soya flour, 0.02% KH_2PO_4, 0.3% NaCl and 0.3% NH_4Cl) were used. In addition, 0.4% $CaCO_3$ was used for anisomycin production.

Table 1
Strains and plasmids used in this study.

Strains or plasmid	Description	Source or Reference
***Streptomyces* strains**		
S. hygrospinosus var. beijingensis	WT anisomycin producing strain	ACCC40033 (Agricultural Culture Collection of China)
Δ*aniF*	*aniF* deletion mutant	[12]
Δ*aniF*-C	*aniF*-complementary strain	This study
O*aniF*	*aniF* overexpression strain	This study
WT::pJTU824	control strain of O*aniF*	This study
Δ*aniF*::pJTU824	control strain of *ΔaniF*	This study
***E. coli* strains**		
DH10B	General molecule cloning strain	Gibco BRL
BL21(DE3)	Host for AniF overexpression	Strategene
ET12567/pUZ8002	Intergeneric conjugation strain	[39]
Plasmids		
pGEX-6p-1	Expression vector	GE Healthcare
pJTU824	Integrating vector containing *ermE** promoter	[40]
pGEX-6p-1-*aniF*	Derivate pGEX-6p-1 plasmid containing the intact gene *aniF*	This study
pJTU824-*aniF*	Derivate plasmid of pJTU824 with intact gene *aniF* under the control of the *ermE** promoter and was used for complementation and overexpression	This study
26D9	The fosmid containing anisomycin biosynthetic gene cluster	[12]

Escherichia coli strains including DH10B, BL21 (DE3), ET12567/pUZ8002 were cultivated at 37 °C in LB and LBBS liquid medium or on LB agar.

2.2. Bioinformatics analysis

The conserved domains were identified by online software NCBI Blastp (https://blast.ncbi.nlm.nih.gov/). The protein secondary structure prediction was conducted by PredictProtein (https://www.predictprotein.org/).

2.3. Gene complementation and overexpression

To construct the complementary strain (*ΔaniF*-C) of *ΔaniF* mutant, a 667-bp DNA fragment carrying the intact *aniF* and two 20-bp homologous arms was amplified by PCR with primers 824-F-fw and 824-F-rv (listed in Table 2). The two homologous arms carried the upstream region containing *Nde*I site and downstream region containing *Eco*RI site, respectively. The PCR product was ligated into the *Nde*I/*Eco*RI-digested pJTU824 generating the recombinant *aniF*-complementary vector pJTU824-*aniF,* using the Ezmax one-step cloning kit (Tolo Biotech, China.). The resultant plasmid was then transferred into *E. coli* ET12567/pUZ8002 and then introduced into *ΔaniF* and *S. hygrospinosus* var. beijingensis via intergeneric conjugation for the construction of complementary strain *ΔaniF*–C and overexpressed strain O*ΔaniF*, respectively.

2.4. Fermentation and detection of anisomycin

S. hygrospinosus var. beijingensis and its derivative strains were cultured on SFM plates (per liter contained 2% agar, 2% mannitol, 2% soybean powder, pH 7.2) at 30 °C for 7 days for sporulation. Then spores were inoculated into 30 mL of TSBY and incubated at 30 °C for another 3 days on a rotary shaker (220 rpm). Seeds were next inoculated into 30 mL fermentation medium (5% (V/V)) and incubated at 30 °C with shaking for further 5–7 days. After that, the fermentation broth was collected by centrifugation (4000 rpm for 30 min). After extraction with equal volume ethyl acetate, the samples were dissolved in methanol and detected by high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) using an Agilent 150*21 mm C18-SB column at 25 °C. The column was equilibrated with 95% solvent A (H_2O with 0.1% trifluoroacetic acid (*v/v*)) and 5% solvent B (methanol) and developed with a linear gradient (5–30 min, from 5% B to 50% B, 30–45 min, from 50% B to 95% B) with a flow rate of 0.5 mL/min and monitored at 223 nm and 280 nm. The quantification of anisomycin was relatively calculated by the peak areas of different fermentation products which used WT as the standard. Each experiment was performed independently in triplicate. LC-MS analysis was conducted with an Agilent 1100 series LC/MSD Trap system with drying gas flow of 10 mL/min, nebulizer at 30 psi, and drying gas temperature of 350 °C.

Table 2
Primers used in this study.

Primers	Sequence (5′-3′)	Use(s)
Gene overexpression and complementation		
F-A-GST	TCCAGGGGCCCCTGGGATCCGTGAAGCG CATATCCGTAAC (*Bam*HI site)	Overexpression of GST-tagged AniF protein in *E. coli*
F-S-GST	TCGAGTCGACCCGGGAATTCTCAGATGTG GCCCTCCCGGA (*Eco*RI site)	
824-F-fw	GTTGGTAGGATCCACATATGGTGAAGCGC ATATCCGTAAC (*Nde*I site)	Complementation of ZXQ09, overexpression of *aniF* in *S. hygrospinosus* var. beijingensis
824-F-rv	TATGACATGATTACGAATTCTCAGATGTGG CCCTCCCGGA (*Eco*RI site)	
EMSAs and DNase I footprinting assays		
aniRG-fw	CGCCAGGGTTTTCCCAGTCACGAC GTTCGCGAGGCGGCTACGGGTG (FAM)	Probe P_{aniR-G}
aniRG-rv	ACGCCGGCGGGCCGTTCGGTCCTG	
aniEF-fw	CGCCAGGGTTTTCCCAGTCACGAC GGGTGTGCTCGGCCGTGGACG (FAM)	Probe P_{aniE-F}
aniEF-rv	GTGAGCGCGGGAACAACCGATG	
Co-transcription analysis		
IH-fw	CGGCAGCGTCGTCACCAAGG	Confirmation that *aniI* and *aniH* are co-transcription
IH-rv	TCCAGCAGGACGTCGAACAG	
JI-fw	GGTCCCCGACGTTCGGCTTC	Confirmation that *aniJ* and *aniI* are co-transcription
JI-rv	CCTTGATCCGGTCCAGTGCG	
ON-fw	AGCGGCTGTTCACCGACTTC	Confirmation that *aniO* and *aniN* are co-transcription
ON-rv	TTGTCGGCGAATCCCGTTGC	
RQ-fw	TGGAACGGAACATCCTCAGCCC	Confirmation that *aniR* and *aniQ* are co-transcription
RQ-rv	AGAGCAGCGAGTCGTCGGAGTG	
RT-qPCR		
16s-RT-fw	CCGCAAGGCTAAAACTCAAA	Detection the transcriptional level of 16s rRNA
16s-RT-rv	AACCCAACATCTCACGACAC	
R-RT-fw	CGCCGCTTCTACCCGCTCAA	Detection the transcriptional level of *aniR*
R-RT-rv	CGGACCACCTCCTGTGCCT	

2.5. Overexpression and purification of recombinant GST-tagged AniF protein

The *aniF* was amplified by PCR with primers F-A-GST/F-S-GST (listed in Table 2). The PCR product was ligated into the *Nde*I/*Eco*RI-digested pGEX-6p-1 with aforementioned homologous recombination kit to generate pGEX-6p-1-*aniF*, which was confirmed by DNA sequencing. The GST-AniF protein was overexpressed in *E. coli* BL21 (DE3). The bacteria were incubated 2% (V/V) in LBBS supplemented with ampicillin (final concentration is 100 μg/mL) for 6 h at 37 °C to OD_{600} 0.6. Isopropylthio-β-D-galactoside (IPTG) with final concentration 0.4 mM was added into the culture and incubated for further 24 h at 16 °C. GST protein encoded by the empty plasmid pGEX-6p-1 was also overexpressed under the same condition as a control. The cells were harvested by centrifugation (3500 rpm, 25 min, 4 °C) and resuspended in PBS (pH 8.0) and lysed by high pressure cracker at 600 bar. Cellular debris was removed by centrifugation (12,500 rpm, 60 min, 4 °C), and the supernatant was used to purify the protein by Glutathione Resin (GenScirpt, China) using standard protocols. The purified AniF-GST and GST proteins were concentrated and stored in 50 mM Tris-HCl (pH 8.0) buffer with 10% glycerol at −80 °C. Protein concentration was determined with the Bradford assay using bovine serum albumin as a standard.

2.6. EMSA

EMSAs were performed according to the procedure described previously [13]. For preparation of 6-carboxyfluorescein (FAM)-labeled probes, FAM-labeled oligos of the promoter regions of *aniF* (365-bp) and *aniR* (293-bp) were PCR amplified with 2× TOLO HIFI DNA polymerase premix (TOLO Biotech, Shanghai) using primers *aniRG*-fw/*aniRG*-rv and *aniEF*-fw/*aniEF*-rv listed in Table 2. The FAM-labeled probes were purified by the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and were quantified with NanoDrop 2000C (Thermo, USA). EMSA was performed in a reaction buffer of 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM DTT, 10% glycerol with 50 ng FAM-labeled probes. Various concentrations of GST-AniF (2–16 μg) were added into the system, while the GST was used as control. Meanwhile, 2 μg salmon sperm DNA was also included in the reaction system for the elimination of the non-specific binding. After incubation for 30 min at 25 °C, the reaction system was loaded into 2% TBE gel buffered with 0.5 × TBE.

In the compound competition experiment, the reaction system (total volume of 20 μL) was prepared the same as the aforementioned and the compounds **3**, **3a**, **4**, **4a** were used with the final concentration of 10 mM. While a gradient concentration of anisomycin was used for the similar assay.

After 120 V pre-electrophoresis in 0.5 × TBE buffer at 4 °C for 30 min, the samples were loaded into 2% TBE gel and continued electrophoresis at 4 °C for 15 min. After electrophoresis, the gels were scanned by ImageQuant LAS 4000 mini (GE Healthcare, America).

2.7. DNase I footprinting assay

DNase I footprinting assays were performed according to the method of Wang et al. [14]. For each assay, FAM-labeled probe (350 ng) was incubated with different amounts of GST-AniF protein in a total volume of 40 μL in the same buffer as that described above for EMSAs. The probe was amplified with the primer pair (*aniRG*-fw/*aniRG*-rv) showed in Table 2. After incubation for 20 min at 30 °C, 10 μL solution containing about 0.015 units DNase I (Promega, America) and 100 nmol freshly prepared $CaCl_2$ were added and further incubated at 37 °C for 1 min. The reaction was quenched by the addition of 140 μL DNase I stop solution, which contained 200 mM unbuffered sodium acetate, 30 mM EDTA and 0.15% sodium dodecyl sulfate (SDS). Mixtures was firstly extracted with phenol/chloroform, and then precipitated with ethanol. Pellets were dissolved in 30 μL MiniQ water. The preparation of the DNA ladder, electrophoresis and data analysis were performed referring to the method described before [14], except that the GeneScan-LIZ600 size standard (Applied Biosystems, America) was used.

2.8. RNA isolation, co-transcription analysis and RT-qPCR

The fermentation cultures of WT and Δ*aniF* mutant strains were collected at 48 h and 72 h, respectively. RNA samples were extracted following TRIzol (SBS, China) according to the manufacturer's instructions. To exclude the genomic DNA's interference, RNA extractions were digested with DNase I (Thermo, America). The clean 1 μg RNA samples were reverse transcribed with reverse transcription system (Thermo, America) according to the procedure recommended by the manufacturer.

4 pairs of primers listed in Table 2 were synthesized and PCR amplification was used to amplify the intergenic region for the analysis of the transcription units within the gene cluster. The fosmid 26D9 was used as positive control. The results were analyzed by agarose gel electrophoresis. The reverse transcription polymerase chain reaction (RT-PCR) was performed using primers listed in Table 2 to identify the transcriptional units. RT-qPCR system was prepared using a SYBR green PCR premix kit (YESEN, China). The PCR procedure contained the following three stages. The first was holding stage which including 50 °C for 20 s, 95 °C for 10 min. The second was cycling stage which including 95 °C for 15 s, 60 °C for 1 min and cycling 40 times. The last stage was melt curve stage which including 95 °C for 15 s, 60 °C for 1 min, 95 °C for 30 s, 60 °C for 15 s. The 16s rRNA was used as an internal control to calculate the transcriptional level of structure gene by the $2^{-\Delta\Delta CT}$ method [15]. Each experiment was performed independently in triplicate.

2.9. Multiple sequence alignment

Multiple sequence alignment was conducted using BioEdit software and the referred homologous proteins were shown as bellow. PapR6 (CBW45649.1) from *Streptomyces pristinaespiralis*, RedZ (CAA69209.1) from *Streptomyces coelicolor* A3(2), DnrN (AAD15247.1) from *Streptomyces peucetius*, VioR (AAP925093f.1) from *Streptomyces vinaceus*, VmsT (BAF50712.1) from *Streptomyces pristinaespiralis*.

2.10. Phylogenetic analysis

Multiple sequences were firstly aligned using ClustalW and the phylogenetic tree of AniF with other LuxR regulators was generated by MEGA (Version 5.05) using neighbor-joining with Poisson correction and 1000 replicate bootstrap analysis. Proteins used for the construction of the phylogenetic tree were: AbsA2 (AAB08053.1) from *Streptomyces coelicolor*, CinR (CAD60529.1) from *Streptomyces cinnamoneus*, ChiR (CAB94548.1) from *Streptomyces coelicolor* A3(2), NcnR (AAD20272.1) from *Streptomyces arenae*, PinM (CAM35468.1) from *Streptomyces natalensis*, SlnM (AHB62093.1) from *Streptomyces lydicus*, SlgR2 (CBA11556.1) from *Streptomyces lydicus*, PldR (BAH02275.1) from *Streptomyces platensis*, TrdH (ADY38540.1) from *Streptomyces* sp. SCSIO 1666, GdmRI (ABI93791.1) from *Streptomyces hygroscopicus*, PikD (AAC68887.1) from *Streptomyces venezuelae*, CarR (AAC38403.1) from *Pectobacterium carotovorum*, EchR (AAA86840.1) from *Dickeya chrysanthemi*, EsaR (KWV85031.1) from *Pseudomonas fluorescens*, TraR (AAD31600.1) from *Agrobacterium radiobacter* K84, LasR (BAA06489.1) from *Pseudomonas aeruginosa*, LuxR (EHN68297.1) from *Vibrio fischeri* SR5.

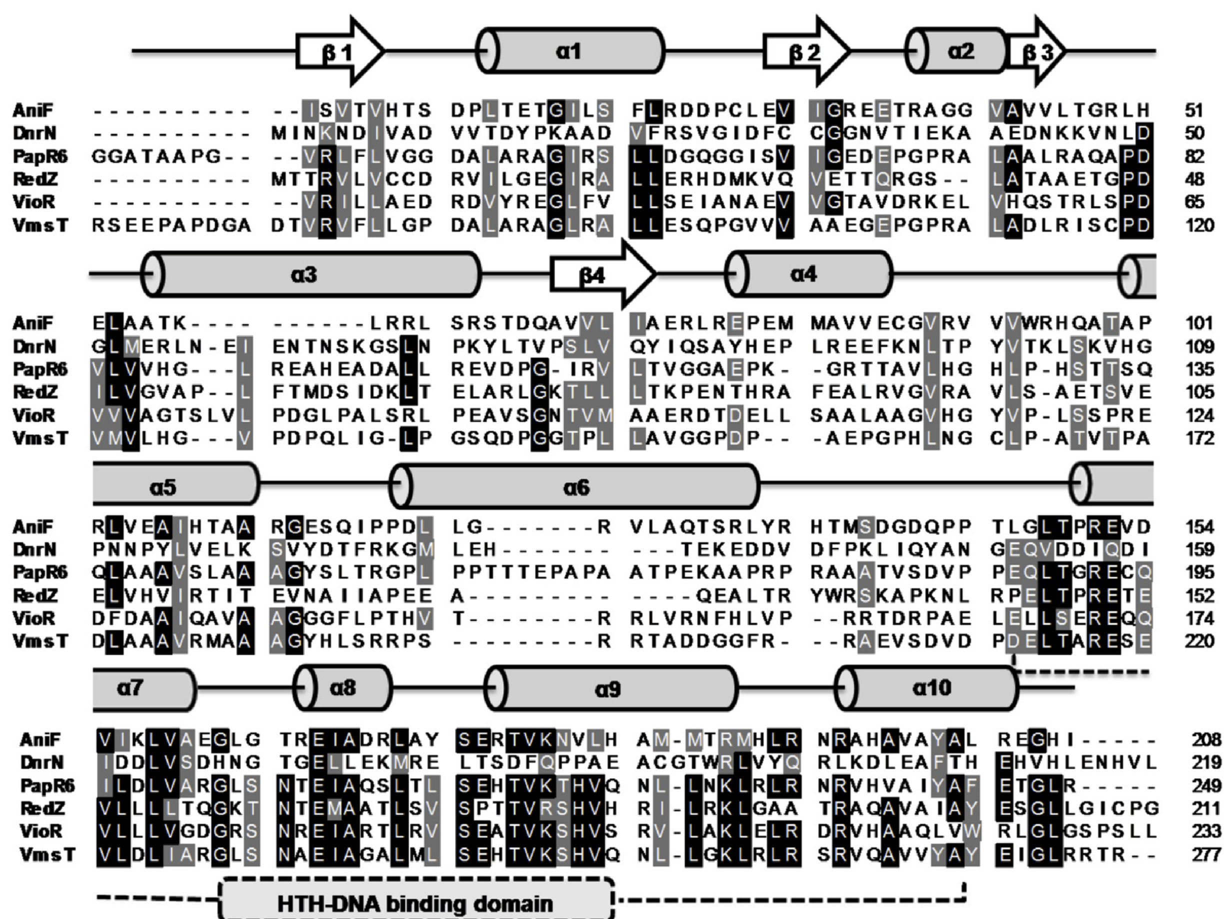

Fig. 2. Sequence alignment of AniF with other atypical LuxR family regulators. The secondary structure element α-helix and β-sheet were indicated by cylinders and arrows, respectively. The putative HTH DNA-binding domain (residues 146–202) was underlined by dotted lines.

3. Results

3.1. AniF activates the production of anisomycin and its derivates

The previous genetic deletion of *aniF* has completely abolished the production of anisomycin and its biosynthetic intermediates (Fig. 1b), suggesting its indispensability [12]. To exclude other possible explanation of the phenotype of *ΔaniF*, a single copy of *aniF* on integrative plasmid pJTU824 (pJTU824-*aniF*) was transferred into *ΔaniF* strain for the construction of the complementary strain (*ΔaniF*-C) (Fig. S1). The fermentation product was firstly qualitatively analyzed by LC-MS (Fig. S2) and the mass/charge (*m/z*) signal is consistent with previous study [12]. The production of anisomycin in *ΔaniF*-C strain was restored, deducting the effect of empty plasmid. This result confirmed the indispensability of *aniF* (Fig. 1b). It is known that the typical way to support a possible regulatory gene is the deletion and the overexpression in combination with the productivity analysis. In order to give further support for the positive regulatory role of AniF, the *aniF*-overexpressing strain O*aniF* was constructed by the incorporation of the aforementioned pJTU824-*aniF* plasmid into the WT strain. After excluding the productivity change exerted by empty plasmid, the anisomycin production in O*aniF* is 1.2 times of WT strain (Fig. 1c). As other three anisomycin analogues have been isolated in *S. hygrospinosus* var. beijingensis, the quantitative analysis was also conducted to explore whether the overexpression of *aniF* showed any positive effect on the improvement of other compounds production. From the data shown in Fig. 1d, the production of deactylanisomycin, component III and component IV in O*aniF* strain was also improved, and the yield was up to 2.9, 2.1 and 3.4 times higher than that in WT strain, respectively. Above all, these results demonstrated that AniF plays a positive regulatory role in anisomycin production and *aniF* could be used for construction of engineering high-yield strains.

3.2. aniF encodes a putative LuxR family transcriptional regulator

The *aniF* gene contains 627 nucleotides and encodes a 208-amino-acid protein AniF. The conducted Blastp bioinformatics analysis of AniF suggested that it putatively belongs to LuxR family transcriptional regulators, possessing the typical LuxR-type helix–turn–helix (HTH)-DNA binding motif [12]. The first protein of this family was reported involved in the typical quorum-sensing (QS) circuit of the symbiotic organism *Vibrio fischeri*, with the presence of autoinducer synthase LuxI [16]. Typically, QS involves the production of N-acyl homoserine lactones by a LuxI homolog and the sensing by the LuxR regulator in a cell-density dependent manner to regulate target genes [17]. Recent studies have uncovered a new group of LuxR regulators that occur without the cognate LuxI homolog, and these regulators are referred to as LuxR orphans or solos, constituting one component system [18]. Compared to the well-studied QS model in Gram-negative bacteria, the LuxR family of proteins has been seldom described in the Gram-positive organisms. Two main groups of LuxR regulators in *Actinobacteria* have been grouped; one that carries a single LuxR domain acts as the transcription factor and another one carries an extra domain related to signal recognition/transduction, besides the typical LuxR domain [19]. The subsequent evolution of these two groups occurred through a series of gene fusion/fission and duplication events, as the result of an overall need to uncouple the signal sensor from the response regulation [19]. This has contributed to the diversification of LuxR family of regulators,

such as proteins containing the PAS (named after homology to the Drosophila period protein (Per), the aryl hydrocarbon receptor nuclear translocator protein (ARNT) and the Drosophila single-minded protein (Sim)) domain (like PimM) [20], regulators containing large ATP-binding regulators of LuxR family (LAL) domain (like PikD) [21] and the CitB subfamily of LuxR proteins (like DnrN) [22]. In the natural product biosynthesis, most of the LuxR family of transcriptional regulators acts as a positive regulator, and a few function as repressor such as AbsA2 [23]. AniF exhibited high sequence identity with CitB superfamily LuxR regulators RedZ (30%) [24] and PapR6 (44%) [25] (Fig. 2). These proteins contain C-LuxR-type HTH domain and N- receiver (REC) domain, and were then sub-grouped into the two components system with phosphorylation site and the one component system without phosphorylation site. The characterized two-component regulatory system is usually composed of a LuxR protein and a sensor histidine kinase (HK) [26]. However, no cognate HK genes was found in close proximity of *aniF* within the anisomycin BGC and the conserved residues in the REC domain for phosphorylation were not identified in AniF. All these data contributed to the assignment of AniF as a putative atypical orphan response regulator (ARR) [27]. This finding will facilitate the future manipulation of *aniF* for engineering transcriptional control.

3.3. AniF activates the transcription of the aniR-G operon

It was previously reported that the BGC of anisomycin contains 13 genes (Fig. 3a). Prior to the identification of target genes regulated by *aniF*, the co-transcriptional analysis was applied to verify the number of operons within this BGC. RT-PCR was performed using primers (listed in Table 2) to detect mRNA spanning different open reading frames (ORFs). All the intergenic gaps between neighboring genes with the same orientation were tested (Fig. 3b). As the gene *aniF* is in the opposite direction to the downstream gene *aniG*, *aniF* was proposed to be transcribed separately. The used RNA here was isolated from the WT strain incubated in the fermentation medium for 48 h, at which time point the anisomycin has already been produced. Reverse transcription was conducted to gain cDNA and the fosmid 26D9 carrying the whole anisomycin biosynthesis gene cluster was used as control. The result showed that all the intergenic gaps between the selected pairs (*aniH-I*, *aniI-J*, *aniN-O and aniQ-R*) are positive to RT-PCR amplification. Therefore, all of the genes (*aniR-aniG*) were co-transcribed from the same promoter and the gene cluster of anisomycin is organized into two operons (*aniF* and *aniR-aniG*). RT-qPCR was conducted to further elucidate the activation role of *aniF* in stimulating the production of anisomycin. The used RNAs were isolated from the *ΔaniF* and WT strains grown in fermentation medium for 48 h and 72 h, respectively. The structural gene *aniR* was selected as the representative of the transcription of the *aniR-G* operon. From the data depicted in Fig. 3c, the transcription level of *aniR-G* operon of *ΔaniF* mutant was negligible and much lower than that of the WT strain at both time points. This result indicated that AniF functioned as a pathway-specific activator for anisomycin biosynthesis by controlling the transcription of the *aniR-G* operon.

3.4. AniF binds to the upstream region of aniR promoter region

To determine whether *aniF* directly activates the expression the genes mentioned above through direct interaction with DNA, the *aniF* gene was firstly expressed in *E. coli* BL21 (DE3). However, His_6-tagged AniF protein expressed in the form of inclusion body, which impeding further characterization. Fortunately, after the selection and optimization of protein expression, the AniF could be expressed as GST-tagged soluble recombinant protein and the purified AniF protein was detected by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 4a). Using the purified GST-AniF, the EMSA experiment was performed according to the protocol described before [13]. The DNA probes containing the upstream regions of *aniF* (365-bp) and *aniR-G* (293-bp, spanning the intergenic region between *aniR* and *orf1662*) were prepared as described before [13]. The resultant probes were named as P_{aniF} and P_{aniR-G}, respectively. As can be seen from Fig. 4b, the purified GST-AniF binds to the P_{aniR-G} and generates obvious shifted bands, while the referred GST protein showed no binding activity with the probe. When P_{aniF} was

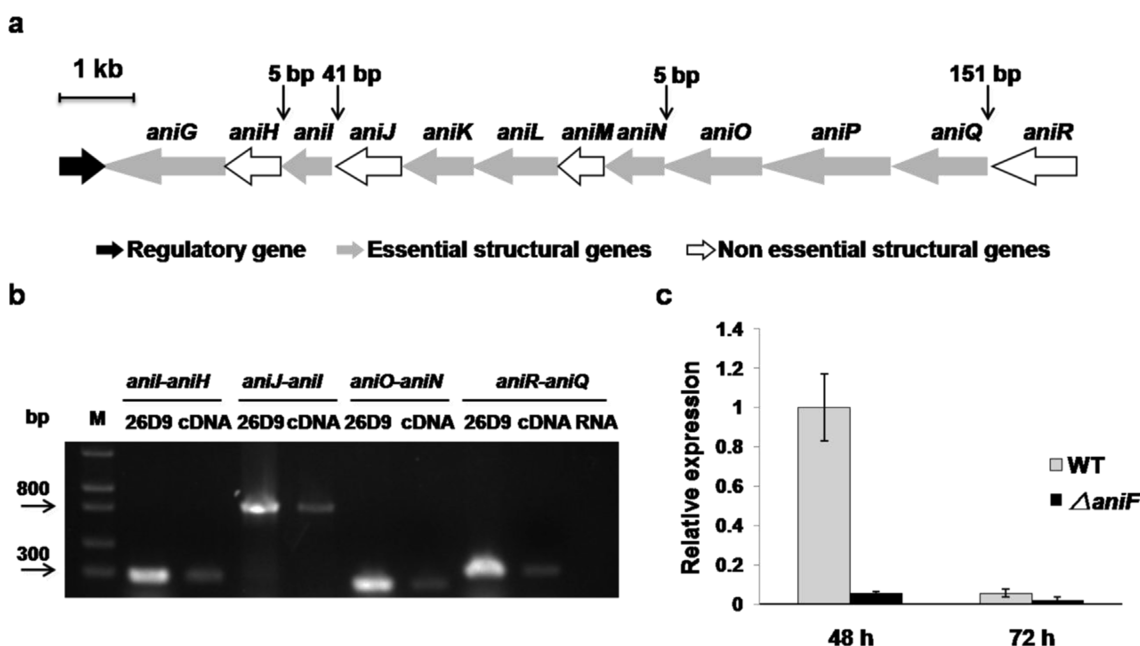

Fig. 3. Co-transcription analysis of anisomycin BGC by RT-PCR and transcriptional levels analysis by RT-qPCR. **(a)** Arrangement of anisomycin BGC. The vertical solid arrows showed the position of primers used for RT-qPCR, and the numbers represent the lengths (bp) of the intergenic regions between adjacent genes. (**b**) Co-transcriptional analysis of anisomycin biosynthetic genes. RNA samples were isolated from WT strain. Fosmid 26D9 containing the whole anisomycin BGC was used as the positive control. RNA was a negative control used as template for amplification of the intergenic region of *aniR-aniQ*. (**c**) The comparative analysis of transcription of the *aniR-G* operon in *ΔaniF* and WT strains.

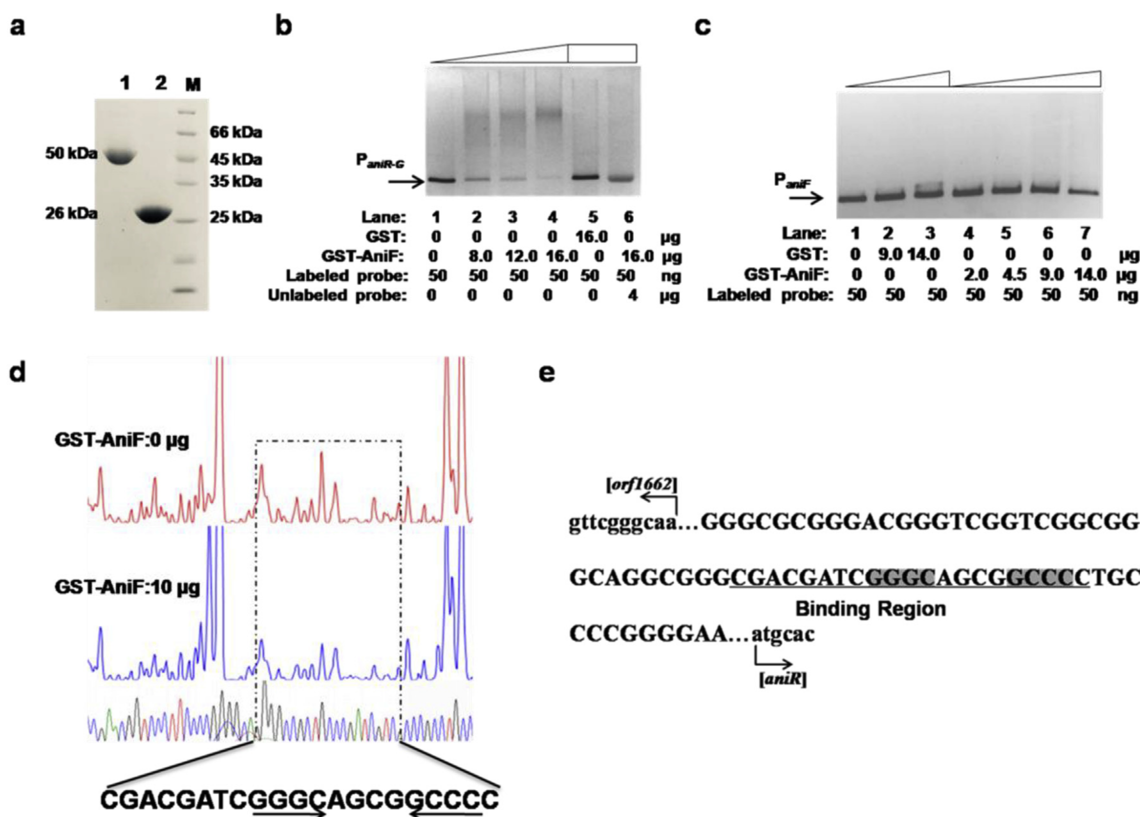

Fig. 4. Binding characters of AniF. (**a**) SDS-PAGE analysis of the purified proteins GST-AniF (lane 1) and GST (lane 2) overexpressed in *E. coli.* (**b**) EMSAs for binding of AniF to P_{aniR-G}. The 293-bp FAM-labeled DNA fragment of the upstream region was incubated with increasing concentrations of GST-AniF protein (lanes 2–4; lanes contain 8, 12, 16 μg GST-AniF, respectively). Lane 1, negative control without proteins. The GST protein and the unlabeled probe in lane 5 and 6, respectively, were used as control. The probes are indicated by arrows. (**c**) EMSAs for binding of AniF to P_{aniF}. The 365-bp FAM-labeled DNA fragment of the upstream region was incubated with increasing concentrations of GST-AniF protein (lanes 4–7; lanes contain 2, 4, 5, 9 and 14 μg GST-AniF, respectively). GST protein with different concentration was added in lane 2 and 3, respectively. Lane 1, negative control without proteins. (**d**) Characterization of the direct binding site of GST-AniF by DNase I footprinting. One protected region was indicated. (**e**) Nucleotide sequence of the identified GST-AniF binding site. The binding site was underlined and the two inverted repeats within AniF-binding site were marked with shaded boxes.

used in the incubation system, neither GST-AniF nor GST could exhibit binding activity (Fig. 4c). This result proved that AniF was not an autoregulator. For the further unveiling of the binding region, DNase I footprinting assay with FAM-labeled primers was then conducted. The data shown in Fig. 4d and e uncovered one protected region (5′-CGA CGATCGGGCAGCGGCCCC-3′). After analyzing the characteristic of binding sequence, we found two inverted repeat sequences (in italics) separated by 4 nt (5′-*GGGC*AGCG*GCCC*-3′). Our findings indicated that AniF may directly activate the transcription of the operon *aniR-aniG* through interaction with the promoter region by recruiting the access of RNA polymerase to this promoter region through an unknown mechanism.

Invert repeats within the target binding region has been reported for other LuxR family of regulators [25,28]. To explore the relationship of AniF with other LuxR family proteins, the phylogenetic analysis was conducted. Based on the analysis, it can be seen that AniF represent a distinct phylogenetic clade separate from those CitB subfamily of LuxR proteins such as DnrN [22], RedZ [24] and PapR6 [25] (Fig. 5). It has been reported that the DNA-binding activity of LuxR proteins can be inhibited by the biosynthesized products, such as DnrN [22] and RedZ [24]. However, that feed-back inhibition seems not applied to all of the family proteins, as no inhibitory effect on PapR6 [25] has been detected. In order to testify whether anisomycin can affect the DNA-binding activity of AniF, anisomycin with gradient final concentration was added into the incubation system of AniF and target DNA and the reactions were conducted according to the previous described procedure. As can be seen from Fig. S3, anisomycin did not exhibit obvious effect on the binding activity of AniF, even the final concentration of anisomycin was added up to 25 mM. To explore whether other biosynthetic intermediates could act as ligands, compounds **3**, **3a**, **4** and **4a** was used to conduct similar assay with the final concentration of 10 mM, respectively (Fig. S3). However, none of these compounds affect the binding reaction. These results excluded the possibility of feedback inhibition exerted by anisomycin and known biosynthetic intermediates. However, further illustration of the regulatory mechanism is still needed.

4. Discussion

Bacteria of the genus *Streptomyces* are a particularly abundant source of natural secondary metabolites, providing more than half of medically and pharmaceutically important antimicrobial and antitumor agents [2,29]. The biosynthetic genes of these products usually cluster together, forming into several operons. The grouped operons provide the opportunity to study the regulation of antibiotic biosynthesis at the molecular level, which give further insight into developing ways of increasing the productivities. Transcription regulation is critical for optimizing protein levels targeting specific metabolites and transcriptional control engineering has been proved valuable for titrating protein levels for titratability of target metabolites, and engineering a plethora of gene circuits in synthetic biology [5,30]. Recently, the regulatory genes have been utilized for the construction of high-yield engineering strain and the activation of silent BGCs. Inactivation of the TetR family transcriptional regulator SACE_3446 stimulated the

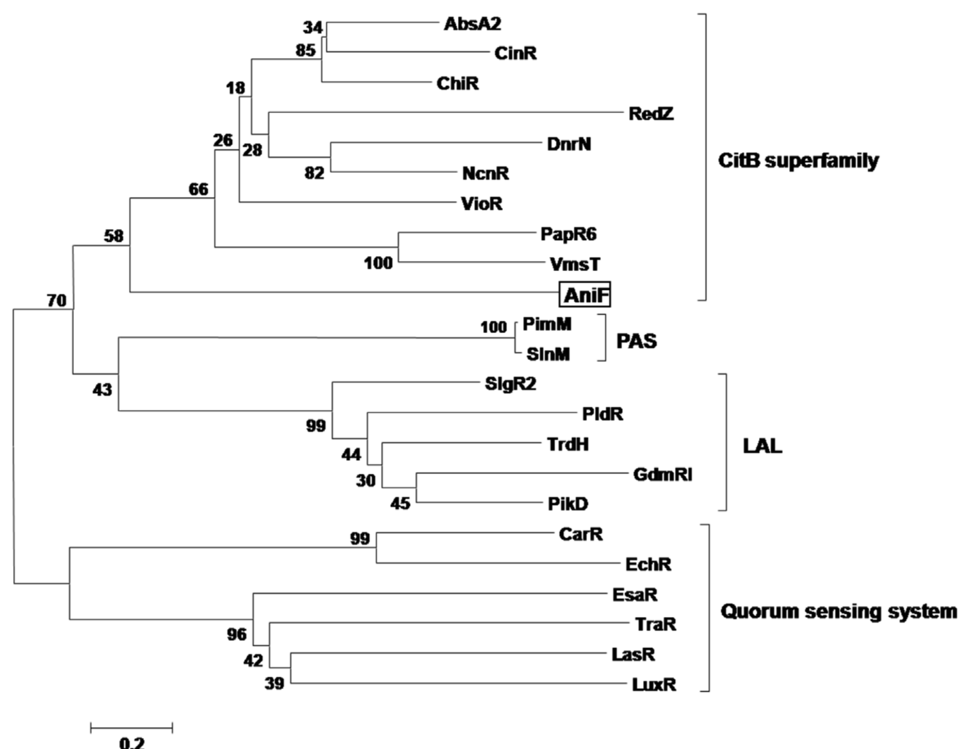

Fig. 5. Phylogenetic analysis of AniF and other LuxR-type regulators.

erythromycin production in *Saccharopolyspora erythraea* and this strategy contributed to significantly elevated production of erythromycin A [31]. The TetR family transcriptional regulator, DepR1, was proved to positively regulate the daptomycin production in the industrial producer *Streptomyces roseosporus* SW0702 [32]. Actually, the genetic engineering by overexpression of *depR1* improved daptomycin production and shortened the fermentation period both in flask and in fermentor. Additionally, the MarR family regulator OtrR was selected for the construction of an inducible expression system, which was used to activate the expression of the BGC of jadomycin [33]. However, more intensive detailed study of regulatory proteins is needed to that end.

Little is known regarding the regulation of anisomycin production at the transcription level, although the biosynthetic pathway has been extensively studied recently in *S. hygrospinosus* var. beijingensis [12]. Previously, the genetic deletion of *aniF* has confirmed its impact on anisomycin biosynthesis and proved its indispensability. However, the regulatory function of it remains to be uncovered. In this study, the complementation of *aniF* in *ΔaniF* mutant successfully restored the production of anisomycin (Fig. 1d). Especially, the overexpression of *aniF* contributed not only to the promoted production of anisomycin but also to the obvious increase in the accumulation of its derivatives deactylanisomycin, component III and component IV (1.2, 2.9, 2.1 and 3.4 fold, respectively) (Fig. 1d). All of these data provided direct support for the positive regulatory role of AniF, and suggested that the manipulation of *aniF* would be an efficient approach for the construction of engineering strain with improved production of anisomycin and its derivatives. Transcription experiments with RT-PCR have shown that AniF stimulate the anisomycin production at the transcriptional level by activating the expression of biosynthetic genes *aniR-G*, covering all of the genes except for *aniF*. Meanwhile, the binding characterization revealed a 21-nt binding region (5′-CGACGATCGGGCAGC GGCCCC-3′) in the upstream region of *aniR*. The sequence analysis revealed that the binding site contains two inverted repeats (5′-GGGC-4 nt-GCCC-3′). Taken together, AniF acted as a pathway-specific transcriptional regulator of anisomycin biosynthesis.

Informatics analysis of AniF has pinpointed it as a potential LuxR family regulator. The most well studied LuxR family DNA-binding proteins are those illustrated involving in two-component quorum-sensing system. Some LuxR-type regulators are activated when bound to autoinducer molecules (N-acyl homoserine cyclic lactones), which is synthesized by LuxI-type proteins. The typical LuxR/LuxI system such as *lasR/lasI* and *traR/traI*, are almost derived from Gram-positive bacteria [34,35]. Subsequently, the divergence and evolution resulted into the various subfamily of LuxR proteins with the varied domain at the N terminal and the conserved LuxR-HTH domain at the C terminal [19], which has been reported as regulators for antibiotic biosynthesis. The LALs contain an N-terminal ATP/GTP-binding domain and a C-terminal LuxR-HTH domain. GdmRI, representative protein of LALs, has been reported to act as the positive regulator required for geldanamycin biosynthesis [36]. The PAS-LuxR regulators, containing the N-terminal PAS domain and a C-terminal HTH motif of the LuxR type, have been shown involved in the biosynthesis regulatory. PimM has been characterized as a transcriptional activator for pimaricin biosynthesis in *Streptomyces natalensis* [20] and SlnM has been proved to promote natamycin production in *Streptomyces lydicus* A02 by the overexpression of its gene [37]. The diverse LuxR family proteins exhibited complex and complicated regulatory mechanism. AniF possesses a REC domain in the N terminal and the classic HTH domain in the C terminal, but not all of the previous reported conserved amino acids within REC domain are clearly annotated. The phylogenetic analysis (Fig. 5) showed that AniF clustered into the same clade with other CitB superfamily response regulators, which contain REC domain with or without conserved phosphorylated sites and LuxR-type HTH domain (Fig. 5). Other CitB superfamily regulators have been reported, such as RedZ and DnrN. RedZ regulated undecylprodigiosin biosynthesis by activating downstream regulator RedD and the binding activity of RedZ with the target DNA could be inhibited by undecylprodigiosin [38]. Similarly, DnrN has been proved to be essential for daunorubin biosynthesis for the transcription of the activator DnrI, and the final product daunorubin appeared to inhibit the binding of DnrN to the promoter of *dnrI* [22]. The function studies of these two proteins provide a foundation of the negative feed repression by final products. However, not all of the CitB

superfamily regulators could be inhibited, like PapR6 [25]. To explore whether the binding activity of AniF could be affected by small molecule ligands, several known biosynthetic intermediates (**3**, **3a**, **4**, **4a**) and the final product anisomycin were added into the incubation system of AniF and target DNA. The data showed that anisomycin and all the biosynthetic intermediates showed negligible effect on the binding activity of AniF (Fig. S3). Other possible specific ligands modulating the DNA-binding activity of AniF remain to be identified, which will contribute to the detailed illustration of the actual regulatory mechanism. Findings reported here may improve the understanding of anisomycin biosynthesis and might benefit the construction of engineering strains for the production of valuable intermediates in response to pharmaceutical development.

Compliance to ethical standards

Conflict of interest

The authors declare that they have no conflicts of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (31630002, 31700029, 31770038, 31470183, 21661140002 and 31170085); the Ministry of Science and Technology, China; Shanghai Pujiang Program from the Shanghai Municipal Council of Science and Technology (12PJD021); and China Postdoctoral Science Foundation (2017M620151).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2018.12.004.

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