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Characterization of the positive SARP family regulator PieR for improving piericidin A1 production in *Streptomyces piomogeues* var. Hangzhouwanensis

Yan Li[1], Lingxin Kong[1], Jufang Shen, Qing Wang, Qian Liu, Weinan Yang, Zixin Deng, Delin You*

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

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ABSTRACT

Piericidin A1, a member of α-pyridone antibiotic, exhibits various biological activities such as antimicrobial, antifungal, and antitumor properties and possesses potent respiration-inhibitory activity against insects due to its competitive binding capacity to mitochondrial complex I. The biosynthetic pathway of piericidin A1 has been reported in *Streptomyces piomogeues* var. Hangzhouwanensis, while the regulatory mechanism remains poorly understood. In this study, a *Streptomyces* antibiotic regulatory protein (SARP) family transcriptional regulator PieR was characterized. Genetic disruption and complementation manipulations revealed that PieR positively regulated the production of piericidin A1. Moreover, the overexpression of *pieR* contributed to the improvement of piericidin A1 productivity. The real-time quantitative PCR (RT-qPCR) was carried out and the data showed that *pieR* stimulated the transcription of all the biosynthesis-related genes for piericidin A1. In order to explore the regulatory mechanism, electrophoresis mobility shift assays (EMSA) and DNase I footprinting experiments have been conducted. A protected region covering 50 nucleotides within the upstream region of *pieR* was identified and two 5-nt direct repeat sequences (5′-CCGGA-3′) in the protected region were found. These findings, taken together, set stage for transcriptional control engineering in the view of optimizing piericidin A1 production and thus provide a viable potent route for the construction of strains with high productivity.

1. Introduction

Microbial natural products have been studied for hundreds of years because of the multiple biological activities and potent pharmaceutical potential that can be used in agriculture, graziery and chemical industry [1,2]. *Streptomyces* are well-known producers of tremendous biological active compounds, and serve as powerful and potent source of important pharmaceutical candidates [3]. α-pyridone antibiotic was first discovered at 19th century by Tuson from castor bean, named ricinine [4]. Subsequently, a series of α-pyridone natural products have been discovered with various biological activities including pesticidal, antifungal, antimalarial and antineoplastic [5,6]. The biosynthesis of these products often assembles with other large biosynthetic machineries, such as polyketide synthase (PKS) and nonribosomal peptide synthase (NRPS) [7]. However, biosynthetic mechanisms of pyridone-based natural products have not been fully revealed.

Piericidins are a family of α-pyridone antibiotics that are isolated mainly from various Streptomyces species of terrestrial, marine, and symbiotic origins [8–10]. Structurally, piericidins feature a pyridone core attached with variable polyene side chains. Piericidin A1, the prototypical member of piericidins, was firstly isolated from *Streptomyces mobaraensis* in the late 1950s, and then was also isolated from other *Streptomyces* [9]. Because of the structural similarity to ubiquinone, piericidin A1 exhibits potent inhibitory activity toward mitochondrial NADH dehydrogenase [11]. Meanwhile, it shows diverse antibacterial and antifungal activities and can selectively kill some insects [12]. Recently, it has been identified as a highly selective antitumor agent in animal model [13]. During the screening for antitumor agents, piericidin A1 has been shown to reduce the resistance of tumor cell against intracellular toxicity and anti-tumor therapeutic agents by inhibiting stress protein GRP78 [13], and inhibit the growth of filopodia protrusion of human epidermal carcinoma A431 cells combined with glucopiericidin A [14]. Moreover, piericidin A is proved to be associated with the quorum sensing of *Chromobacterium violaceum* CV026 strain related to potato soft rot [15].

Since the first isolation, the total complex chemical synthesis of it

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* Corresponding author.
E-mail address: dlyou@sjtu.edu.cn (D. You).
[1] These authors contributed equally to this work.

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has been achieved successfully [16], nevertheless, alternative more efficient green approaches are needed. Previous study has contributed to the identification of the biosynthetic gene cluster (*pie* cluster) of piericidin A1 in *Streptomyces piomogeues* var. Hangzhouwanensis and the reveal of its biosynthesis pathway [17]. The α-pyridone ring formation is dependent on hydrolysis of the linear β,δ-diketo carboxylic acid synthesized by type I polyketide synthases, followed by amidation and cyclization. This was strikingly different from the previously characterized hybrid polyketide synthase-nonribosomal peptide synthetase (PKS-NRPS) involved in α-pyridone pathways. Three post-modification proteins encoded in the gene cluster were proved responsible for the hydroxylation and methylation in piericidin A1 [18]. The production of antibiotics in *Streptomycetes* is usually controlled by multiple regulatory proteins that respond to internal physiological and environmental conditions. Typically, *Streptomyces* have more than 8000 protein-coding genes, and > 10% of the coding genes are predicted to be transcription factors, exhibiting remarkable regulatory capacity and flexibility [19]. Regulation is critical for optimizing protein levels and the subsequent cellular levels of metabolites [20,21]. Genetic manipulation of regulatory genes has emerged as an important tool for construction of high-yield strains [22–25], while transcriptional control engineering has been proved to be a valuable tool for titrating protein (and therefore activity) levels for titratability of metabolites of interest, and engineering a plethora of gene circuits [21] in synthetic biology.

In order to explore the biosynthetic regulatory mechanism and provide insight into future molecular synthetic engineering construction of piericidin A1 and its derivates, the regulatory role of PieR from *Streptomyces piomogeues* var. Hangzhouwanensis was characterized in this study. The bioinformatic analysis of PieR has revealed that it is a possible member of *Streptomyces* antibiotic regulatory protein (SARP) family of transcriptional regulators. In this study, the genetic manipulation of *pieR* proved its positive stimulation on piericidin A1 biosynthesis and the overexpression of *pieR* resulted in a 2.3-fold improvement of piericidin A1 productivity than wild type (WT) strain. Meanwhile, the target gene of PieR was identified by EMSA and the PieR-binding sequence was determined by DNase I footprinting. The findings reported here showed that PieR appeared to promote the expression of the *pie* cluster and in turn contributed to further accumulation of piericidin A1.

2. Materials and methods

2.1. Strains and general techniques

Strains and plasmids used in this study were listed in Table 1. *S. piomogeues* var. Hangzhouwanensis, the wild type producer of piericidin A1, was used as original strain for construction of *pieR* interruption and over-expression mutants. *Escherichia coli* strain BW25113 was used for the construction of *pieR* mutant, which was manipulated according to the previous protocol [26]. *E. coli* strain DH10B was used for general cloning [27]. *E. coli* strain ET12567 carrying plasmid pUZ8002 was used for conjugation with *Streptomyces* [28]. *E. coli* BL21 (DE3)/pLysE was used as host for protein expression. pET28a (Novagen) was used as protein expression vectors. pIJ778 was used as template for the amplification of *aadA* + *oriT* cassette for the disruption of *pieR*. The integrative plasmid pSET152 was used for gene complementation. General genetic manipulation of *E. coli* or *Streptomyces* were carried out according to the reported procedure [29,30].

2.2. Construction of gene disruption, complementation and over-expression mutants

The disruption of *pieR* was conducted using PCR targeting system based on homologous recombination [26]. Plasmid pIJ778 was used as template for PCR amplification of the 1.4 kb gene disruption *aadA* + *oriT* cassette. The resultant fragment was electroporated into *E. coli* BW25113/fosmid 7D9 (containing the whole *pie* gene cluster) for the replacement of 462 bp of *pieR*. The positive mutant was verified by PCR and then, was introduced into *S. piomogeues* var. Hangzhouwanensis by intergeneric conjugation. The double-crossover strain *ΔpieR* was obtained from antibiotic selection (spectinomycin) on YMS solid medium and was further confirmed by PCR. The pSET152 derivate plasmid containing the intact *pieR* was introduced into the *ΔpieR* and wide type strain for the construction of complementary and overexpression strain, respectively. All the primers used here were listed in Table 2.

2.3. Fermentation and detection of piericidin A1

Spores were inoculated into YEME medium (yeast extract, 3 g/l; tryptone, 5 g/l; maltose, 3 g/l; glucose, 10 g/l; sucrose, 103 g/l) in the proportion of 0.1% and cultivated at 30 °C for 3 days. Then, 5 ml seed broth was inoculated into 100 ml fermentation medium and cultivated at 30 °C for another 3 days [17]. After fermentation, add 100 ml acetone into fermentation broth and shake for 12 h to disrupt cell. Next, centrifuge fermentation broth and evaporate the supernatant. After acetone is evaporated, add equally volume of ethyl acetate to extract piericidin A1 twice. Finally, evaporate ethyl acetate to dryness and dissolve the extract in 1 ml methanol. The process of extract and evaporate was conducted in the dark environment in case of the degradation of piericidin A1 [7]. Before detection, the extract was diluted 10 times with methanol and filtrated with 0.22 μm membrane. The resultant methanol extract was analyzed by Agilent HPLC series 1100 with an Agilent ZORBAX SB-C18 column (5 μm, 4.6 × 250 mm). The column was equilibrated with 80% solvent A (H_2O) and 20% solvent B (acetonitrile) and developed with a linear gradient (5–35 min, from 20% B to 80% B, 35–40 min, from 80% B to 100% B) and then kept 100% B for 5 min at a flow rate of 0.6 ml/min and UV detection at 232 nm. LC-MS analysis was conducted with Agilent 1100 series LC/MSD Trap system with drying gas flow 10 ml/min, nebulizer 30 psi and drying gas temperature 350 °C. Pure piericidin A1 standard was used as control.

2.4. Growth measurement

Spores were inoculated as described above. 1 ml culture was collected at different time point (0, 3, 6, 9, 12, 24, 36, 48 h) to monitor the OD_{600} for the depiction of growth curve and another 1 ml culture was centrifuged and dried at 65 °C for biomass measurement.

2.5. RNA isolation, co-transcription analysis and real-time quantitative PCR (RT-qPCR)

1 ml fermentation culture was collected and washed by 1 ml water. After treated with lysozyme for 2 h, cells were disrupted by magnetic beads with 1 ml Redzol (sbs). Then, RNA was extracted according to the procedure provided by manufacturer. The quality of RNA was detected by NanoDrop 2000C (Thermo, USA). Reverse transcription was conducted before co-transcription analysis. Extracted RNA was firstly treated with DNase I for 4 h to eliminate gDNA. Then, reverse transcription experiment was carried out using Revert Aid Minus Fist Strand cDNA Synthesis Kit (Thermo Scientific). 12 pairs of primer listed in Table 2 were synthesized and PCR amplification was used to amplify the intergenic region. Genomic DNA of wild type strain was used as a control. 16s rRNA gene and *pieE* gene was used as internal reference. The results were analyzed by agarose gel electrophoresis.

RT-qPCR experiment was conducted by 7500 Fast Real-Time PCR System using SYBR Green Master Mix (YEASEN). The transcription level of gene was analyzed according to comparative CT (ΔΔCT).

2.6. Expression and purification of PieR

The primers for cloning *pieR* were listed in Table 2. After amplified by PCR and digested with *Nde*I and *Eco*R I, *pieR* was cloned into pET-

Table 1
Strains and plasmids used in this study.

Strain or plasmid	Description	Source
Strains		
Streptomyces		
S. piomogeues var. Hangzhouwanensis	Wild-type strain producing piericidin A1	Zhejiang Academy of Agricultural Sciences
Δ*pieR*	a disruption of *pieR* by PCR targeting	This study
Δ*pieR*-C	Δ*pieR*::pSET152-*pieR*, complemental strain	This study
Δ*pieR*::pSET152	control strain of Δ*pieR*-C	This study
O*pieR*	WT::pSET152-*pieR*, over-expression strain	This study
WT::pSET152	control strain of O*pieR*	This study
E.coli strains		
BW25113	Containing λ RED recombination plasmid	[26,27]
BW25113/fosmid 7D9	Containing fosmid 7D9	This study
ET12567/pUZ8002	Mediating intergenic conjugation	[28]
DH10B	Host for cloning	[27]
BL21(DE3)	Host for protein expression	Stratagene
Plasmids		
pOJ446	*aac (3)IV*, SCP2, *reppMB1**, *attΦC31*, *oriT*	[47]
Fosmid7D9	pOJ446 derivative carrying *pieR-A1* gene	Current study
pLY-1	Fosmid7D9 derivative which *pieR* was partially replaced by spectinomycin resistance gene	This study
pIJ778	Containing spectinomycin resistance marker cat	[26]
pSET152	Integrative plasmid used for complementation	[47]
pLY-2	pSET152 derivative carrying *pieR* gene	This study
pET28a	Protein expression vector	Novagen
pLY-3	pET28a derivative carrying *pieR* gene	This study

Table 2
Primers used in this study.

Primer name	Sequence (5′-3′)
VLY-1R	GGCGAACCTGCACAGCTACGT
VLY-1F	CTCGGCTTTCCTCTTCGTCTCG
16S-R	CATGCAAGTCGAACGGTGAA
16S-F	GCCGTGTCTCAGTCCCAGTG
PieE-R	CCGCTGCTCGCCAACCACAT
PieE-F	ACCAGCAGCGGCTCCATCAG
RA1-R	GGCCGTCCTACGTCCATTCG
RA1-F	AGCGGTGGGTTCCCTCCTTC
A1A2-R	AGGAGGAGGAGCACGGAAGC
A1A2-F	GCGGTTCTGCTGCCGCAGCA
A2A3-R	CGACGAGCTGCTCGACATCG
A2A3-F	ATGGTCAGCCGCTTGAGGGT
A3A4-R	AGCAGTTCCTGACGCCCTAC
A3A4-F	TTCTTGTGCCACTTGAGCCT
A4A5-R	CGTCAGCTGGTGGTTCTGCT
A4A5-F	GGACGACCAACTGGACGAGA
A6B1-R	GGCGACCACTTCTCCCTGAC
A6B1-F	TGGTGGTCGTTGTCGATGCT
B1C-R	GCCCCACCCCTGAAGACCCC
B1C-F	TGAGCTGACGCATGCCCTTG
CD-R	CCTGGACCGTTTCTTCTTCG
CD-F	GTGTCGGTCATGGCGCTCAG
DB2-R	GACCTCTACCGCCCGACCAT
DB2-F	TTGGTCAGCACGTCGTGGAA
B2E-R	GGTGCGCTGGTTCAGCAGGT
B2E-F	TCGGCGTTCACAAGGACACG
pieR-R	AAAGAATTCTCAGGCGGACCCGGCGAGGCG
pieR-F	AAACATATGGTGATTTTCAGTGTTCTTGGCCC

28a. The recombinant plasmid pLY-3 was verified by sequencing. Then, pLY-3 was transformed into *E. coli* strain BL21 (DE3) for PieR expression. The resultant *E. coli* BL21 cell was cultured at 37 °C and 220 rpm in LB medium supplemented with kanamycin (final concentration is 50 g/ml) to OD_{600} = 0.6. Isopropylthio-β-D-galactoside (IPTG) with final concentration 0.4 mM was added into the culture after cooling at 4 °C for 30 min to induce protein expression. The cells were further cultured at 30 °C for 6 h, and then the cells were harvested by centrifugation (×750 g, 25 min, 4 °C) and resuspended in Buffer A (50 mM HEPES, 500 mM NaCl, 10% glycerol, pH 7.0) and lysed by high pressure cracker at 600 bar. Cellular debris was removed by centrifugation (×750 g, 60 min, 4 °C), and the supernatant was used to purify the protein by nickel-affinity chromatography using standard protocols. The protein was eluted with increasing gradient of buffer B (1M imidazole in buffer A). Purified protein was concentrated and exchanged into buffer A with the centrifugal filters (Amicon). The protein was stored in buffer A at −80 °C. Protein concentration was determined with the Bradford assay using bovine serum albumin as a standard.

2.7. Electrophoretic mobility shift assay (EMSA)

For preparation of fluorescence (FAM) labeled probes, FAM-labeled oligos of the promoter regions were PCR amplified with 2x TOLO HIFI DNA polymerase premix (TOLO Biotech, shanghai) using primers of Probe1-F-M13F-47 (FAM) and Probe1-R listed in Table 3. 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 40 ng probes. PieR proteins with final concentration of 0, 2, 5, 10 μg was added, respectively. Meanwhile, 2 μg salmon sperm DNA was also included in the reaction system. After incubation for 30 min at 25 °C, the reaction system was loaded into 2% TBE gel buffered with 0.5 × TBE.

2.8. DNase I footprinting assay

For preparation of fluorescent FAM labeled probes, PCR amplified the promoter region with 2x TOLO HIFI DNA polymerase premix (TOLO Biotech, Shanghai) from the fosmid 7D9 using primers of M13F-47 (FAM) and probe1-R. The FAM-labeled probes were firstly purified by the Wizard® SV Gel and PCR Clean-Up System (Promega, USA), and then were quantified with NanoDrop 2000C (Thermo, USA). DNase I footprinting assays were performed according to the procedures described before Wang et al. [31]. For each assay, 350 ng probes were

Table 3
Probes used in this study.

Probe name	Sequence (5′-3′)
Probe1-F	CGCCAGGGTTTTCCCAGTCACGACGGCCCGCACCAGAGGAATTC
Probe1-R	GGTTTGGTATGGCACGCCCAC
M13F-47	CGCCAGGGTTTTCCCAGTCACGAC

Table 4
Proteins used in the alignment analysis.

Protein	Strains	Identity
ARV85759.1PieR	*Streptomyces philanthi* bv. triangulum	71%
ACE02599.1 AurD	*Streptomyces thioluteus*	54%
AGG68812.1 TylT	*Streptomyces fradiae*	41%
BAA14186.1 AfsR	*Streptomyces coelicolor*	39%
AAV31783.1 SanG	*Streptomyces ansochromogenes*	36%
ACR48345.1 NosP	*Streptomyces actuosus*	33%

incubated with different amounts of proteins (0, 0.6 μg) in a total volume of 40 μl. After incubation at 30 °C for 20 min, 0.015 unit DNase I (Promega) and 100 nmol freshly prepared $CaCl_2$ prepared in another 10 μl solution was added and further incubated at 37 °C for 1 min. The reaction was quenched by adding 140 μl DNase I stop solution (200 mM unbuffered sodium acetate, 30 mM EDTA and 0.15% SDS). Samples were firstly extracted with phenol/chloroform, then precipitated with ethanol. Pellets were dissolved in 30 μl MiniQ water. Methods for preparation of the DNA ladder, electrophoresis and data analysis were the same as described before [31], except that the GeneScan-LIZ600 size standard (Applied Biosystems) was used.

2.9. Multiple sequence alignment and secondary structure prediction

Multiple sequence alignment was conducted using BioEdit software and the referred homologous proteins were listed in Table 4. The prediction of secondary structure of PieR is conducted by PSIPRED v3.3.

2.10. Phylogenetic analysis

Multiple sequences were firstly aligned using ClustalW and the phylogenetic tree of PieR with other SARPs was generated by MEGA (Version 5.10) using neighbor-joining with Poisson correction and 500 replicate bootstrap analysis [32,33]. The detailed information about the selected SARPs listed as bellow. PieR, from *Streptomyces philanthi* bv. triangulum (ARV85759.1); AurD, from *Streptomyces thioluteus* (ACE02599.1); TylT, from *Streptomyces fradiae* (AGG68812.1); AfsR, from *Streptomyces coelicolor* (BAA14186.1); SanG, from *Streptomyces ansochromogenes* (AAV31783.1); NosP, from *Streptomyces actuosus* (ACR48345.1).

3. Results

3.1. pieR encodes a putative SARP family transcriptional regulator

Bioinformatics analysis of PieR revealed that it possessed the conserved DNA binding domain in N terminal that resembles to that of OmpR and bacterial transcription activation domain in C terminal (BTAD domain), which are characteristic of SARP family proteins (Fig. 1). SARPs usually act as pathway-specific regulators directly affecting the transcription of specific gene cluster. Many SARP proteins have been characterized as activators for antibiotics synthesis, such as NosP [34] and PolR [35]. Meanwhile, PieR shows 71% identity with another PieR from another piericidin-producing strain *Streptomyces philanthi* and 54% identity with AurD from *Streptomyces thioluteus* (Table 4). It is located upstream of *pieA1*, which is the first structural gene encoding a putative type I PKS. The secondary structure of PieR was analyzed by PSIPRED. As is shown in Fig. 1, the possible DNA binding domain of PieR covers three α-helixes packed against two antiparallel β-sheets forming the characterized winged helix-turn-helix (HTH) domain [36]. The BTAD domain was proposed to compose seven α-helixes (α4-α10) in accordance with a previous finding [37]. All these data contributed to the assignment of PieR as a typical SARP family transcriptional regulator.

3.2. PieR positively activates the production of piericidin A1

To determine the role of *pieR* in piericidin biosynthesis, *pieR* was disrupted via PCR-targeting system, which has been widely applied to gene disruption in *Streptomyces* (Fig. S1A) and the disruption of *pieR* was verified by PCR (Fig. S1B). To inspect the effect of the *pieR* disruption on the production of piericidin A1, the *ΔpieR* mutant strain and WT strain were cultured in fermentation medium for 3 days and the fermentation product of *ΔpieR* mutant was firstly qualitatively analyzed by ESI-MS (Fig. S2) and the mass/charge (m/z) signal is consistent with previous study [17]. Then, the production was quantitatively compared to that of WT strain by high-performance liquid chromatography (HPLC). As can be seen from Fig. 2A, the yield of piericidin A1 in *ΔpieR* was only 11% of the WT strain, suggesting the positive role of *pieR* in piericidin A1 biosynthesis. To verify that the decreased production of piericidin A1 was directly resulted from the interruption of *pieR*, a single copy of *pieR* on integrative plasmid pSET152 was then transferred into *ΔpieR* strain for the construction of the complementary strain (*ΔpieR*-C). The production of piericidin A1 in *ΔpieR*-C strain nearly restored to that of WT strain, deducting the effect of empty plasmid (Fig. 2B). As the overexpression of positive regulatory genes have been proved as an important method for increasing the production of target natural product, to further consolidate the positive role, a copy of *pieR* was also integrated into the WT strain, resulting in the overexpressing strain (O*pieR*). After excluding the productivity change exerted by empty plasmid, the piericidin A1 production in O*pieR* is up to 2.3 times of WT strain (Fig. 2B). To validate that the productivity changes were only induced by the regulatory role of *pieR*, both of the growth curve and biomass were characterized in the WT and *ΔpieR* strains (Fig. 2C and D). Consistently, the WT and *ΔpieR* strains shared similar characters and exhibited negligible differences. Taken together, these results obviously provided sufficient support for the positive regulatory role of *pieR* in piericidin A1 biosynthesis.

3.3. PieR activates the transcription of the pie cluster

Previous study has revealed that the *pie* cluster contained 12 genes in the same direction (Fig. 3A). In order to verify the number of operons within this cluster and facilitate the transcriptional analysis of all the genes, one-step RT-PCR was performed using primers listed in Table 2 to detect mRNA spanning different ORFs. All the intergenic gaps between neighboring genes with the same orientation were tested (Fig. 3A), excepting the two genes *pieA5* and *pieA6* that overlapped 88 bp. The result showed that all the intergenic gaps are positive to RT-PCR amplification. Therefore, all of the genes were organized into one operon and co-transcribed from the same promoter upstream of *pieR*, forming the *pie* operon (Fig. 3B). To validate that *pieR* affected the production of piericidin A1 by regulating the transcription of *pie* gene cluster, the reverse transcription polymerase chain reaction (RT-PCR) was conducted. The used RNAs were isolated from the *ΔpieR* and WT strains grown in fermentation medium for every 24 h, respectively. The structural gene *pieE* was selected as the representative in the transcription of the *pie* cluster. From the data depicted in Fig. 3C, the transcription level of *pie* operon of *ΔpieR* mutant was much lower than that of the WT strain. The transcriptions of *pie* cluster reached the maximum at the third day in both strains (Fig. 3C). Correspondingly, the time-course analysis of piericidin A1 productivity was also carried out. From Fig. 3D, the yield of piericidin A1 was negligible compared with that of WT strain, and the productivity also reached the highest point at the third day (Fig. 3D). All these data strongly supported that *pieR*, a potent regulatory gene, activated the piericidin A1 biosynthesis at the transcriptional level.

3.4. PieR binds specifically to the upstream region of pieR

Given the above-revealed positive regulatory role of *pieR*, to explore

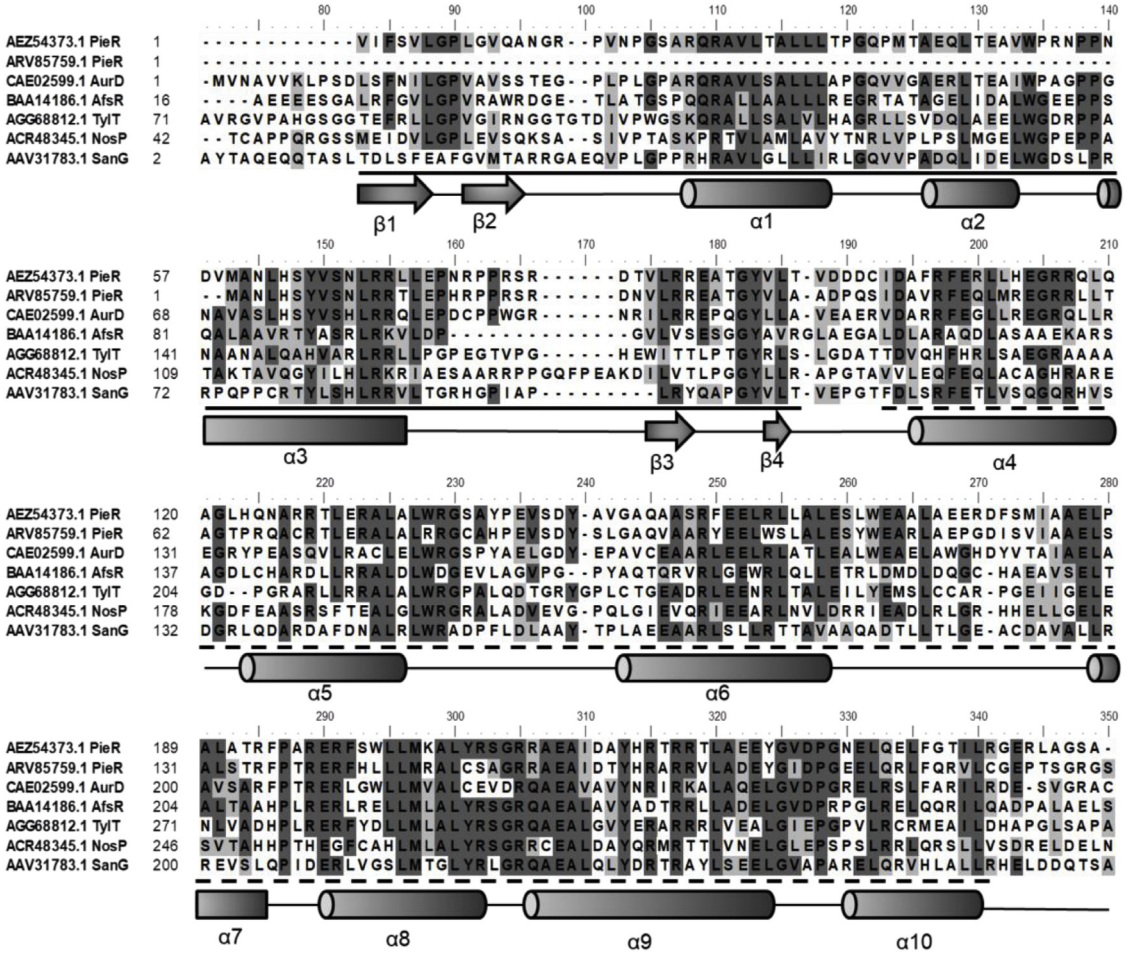

Fig. 1. Sequence alignment of PieR with other homologous SARP proteins. The HTH domain was underlined with solid line and BTAD domain was underlined with dotted line. The secondary structure element α-helix and β-sheet were indicated by cylinders and arrows, respectively.

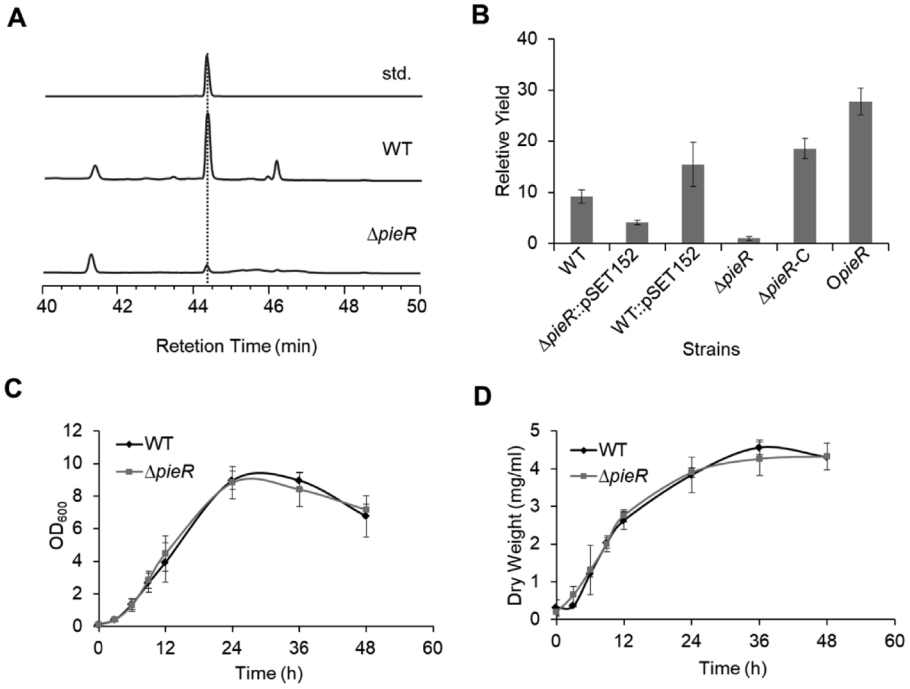

Fig. 2. Effects of deletion and overexpression of pieR on the production of piericidin A1. (A) HPLC analysis of the production of piericidin A1 in WT and *ΔpieR* strains. (B) Quantitative analysis of piericidin A1 in WT, *ΔpieR*, *ΔpieR-C*, *ΔpieR*:pSET152, WT:pSET152-*pieR* and O*pieR* strains. To facilitate the comparison, the productivity of piericidin A1 in *ΔpieR* is determined as 1. (C) Characterization of the growth curve of WT and *ΔpieR* in YEME medium. (D) Biomass assay of WT and *ΔpieR* in YEME medium.

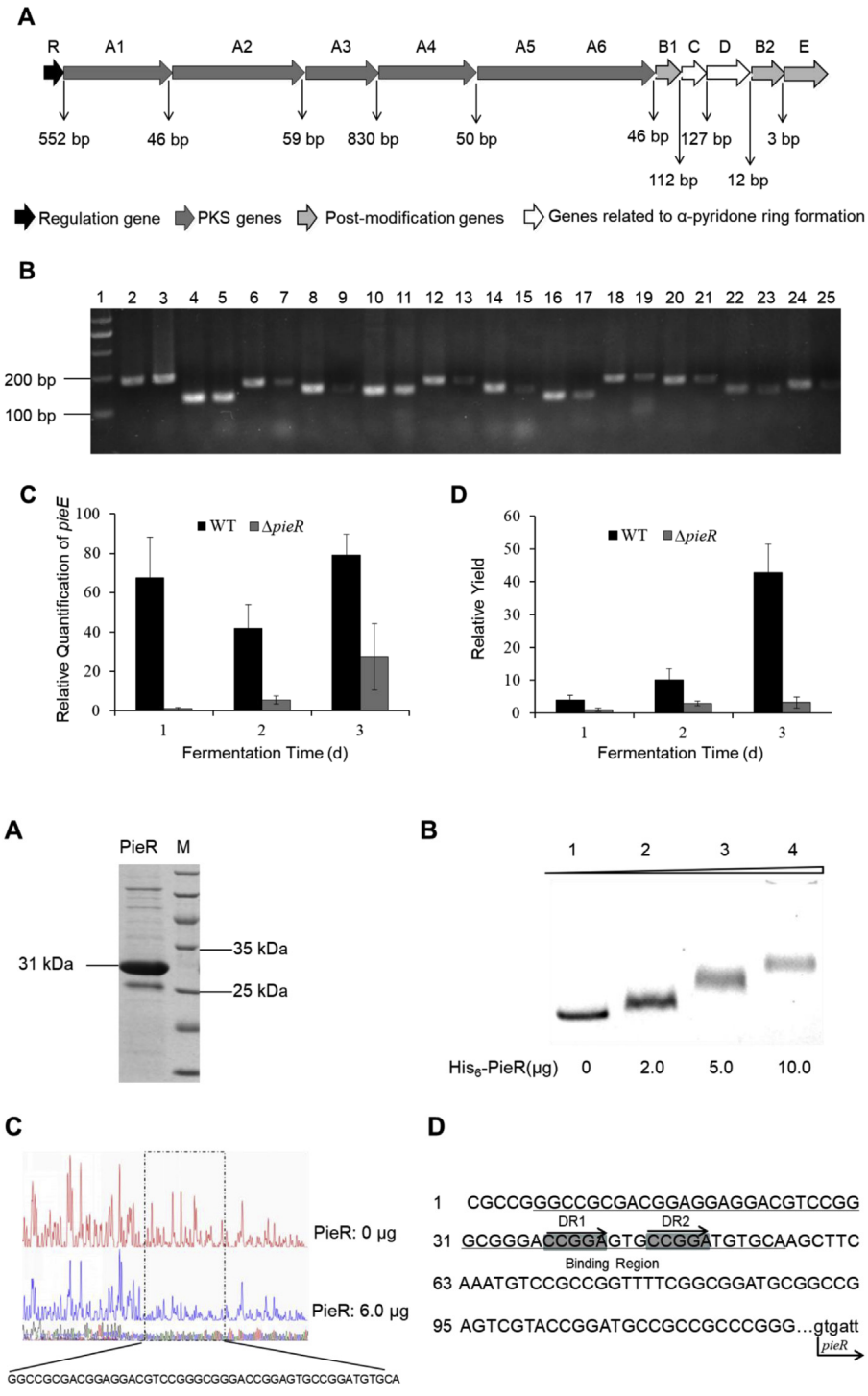

Fig. 3. Transcription analysis of the biosynthetic gene cluster of piericidin A1 and productivity analysis in *ΔpieR* strains. (A) Organization of the genes encoded by *pie* cluster. Genes are assigned in different colors, regulatory gene is marked in black, PKS genes are in dark gray, genes related to α-pyridone ring formation are in white, while post-modification genes are marked in light gray. The vertical solid arrows showed the position of primers used for RT-qPCR, the numbers represent the lengths (bp) of the intergenic regions between adjacent genes. (B) PCR confirmation of the amplicons fragments on an ethidium bromide-dyed agarose gel. The amplicons were designed to cover the adjacent genes, genomic DNA was used as the positive control. Line 1, Marker; Line 2, genomic DNA used as templet to amplify 16s rRNA; Line 3, cDNA used as templet to amplify 16s rRNA; Line 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, genomic DNA used as template for the amplification of *pieE* (partial) and the intergenic regions between *pieR-A1*, *pieA1-A2*, *pieA2-A3*, *pieA3-A4*, *pieA4-A5*, *pieA6-B1*, *pieB1-C*, *pieC-D*, *pieD-B2*, pie*B2-E*, respectively. While line 5, 7, 9, 11, 13, 15, 17,19, 21, 23, 25, cDNA used for the amplification of *pieE* (partial) and the intergenic regions between *pieR-A1*, *pieA1-A2*, *pieA2-A3*, *pieA3-A4*, *pieA4-A5*, *pieA6-B1*, *pieB1-C*, *pieC-D*, *pieD-B2*, *pieB2-E*, respectively. (C) The transcription analysis in *ΔpieR* during fermentation. *pieE* was selected as representative, 16s rRNA gene as reference and the WT strain was used as control. (D) Time-course analysis of piericidin A1 production in *ΔpieR* strains. The yield of it in the WT strain was used as control.

Fig. 4. Binding characters of PieR for controlling of the transcription of *pie* cluster. (A) Purified PieR analyzed by SDS-PAGE. (B) EMSAs for binding of PieR to the upstream region of *pieR*. The 350 bp FAM-labeled DNA fragment (40 ng) of the upstream region was incubated with increasing concentrations of PieR protein (lanes 2–4; lanes contain 2, 5, 10 μg PieR, respectively). Lane 1, negative control without PieR. The shifted bands are indicated by arrows. (C) Characterization of the direct binding site of PieR by DNase I footprinting. Protected region was indicated. (D) Nucleotide sequence of the PieR-binding sites. The PieR-binding sites are underlined and the direct repeats are marked with gray rectangles. The bent arrows indicate the transcription start points and transcription orientation of *pieR*.

the possible regulatory mechanism, *pieR* was next expressed for the identification of target binding region in vitro. When the previously annotated *pieR* gene (Genebank HQ840721.1) was expressed in *E. coli* BL21 (DE3), the protein existed in the form of insoluble inclusion body. The subsequent optimization of culture condition and expressing vectors with different tag failed as well. However, we tried to clone a longer fragment covering the upstream 174 bp and the previously annotated *pieR* gene. This newly cloned fragment (774 bp) began with GTG and was then cloned into the pET28a plasmid for the construction of pLY3. Fortunately, when this recombinant plasmid was expressed in *E. coli* BL21 (DE3), an obvious overexpressed protein band (31 kDa) could be detected in the soluble component. Finally, the expressed product could be purified as a His_6-PieR recombinant protein (the whole sequence analyzed in Fig. 1). The purity of purified protein was analyzed by SDS-PAGE (Fig. 4A) and the concentration was determined by Bradford assay using bovine serum albumin as a standard. Using the purified PieR, the EMSA experiment was performed according to the protocol described before [31]. The fragment of 326 bp upstream of *pie* cluster was amplified by PCR with primers probe1-R(5′-GTTTGGTATGGCACGCCCAC-3′) and probe1-F(5′-CGCCAGGGTTTTCCCAGTCACGACGGGCCCGCACCAGAGGAATTCG-3′), the resultant fragment was then used as template for the preparation of fluorescent probe with primers M13F-47(5′- CGCCAGGGTTTTCCCAGTCACGAC-3′) and probe1-R(5′-GTTTGGTATGGCACGCCCAC-3′). The resultant fluorescent probe

was incubated with purified PieR of gradient concentrations in a typical reaction system, which was composed of 100 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM DTT supplied with 2 μg salmon sperm DNA under room temperature for 30 min. After that, the reaction product was transferred to gel analysis. As was shown in Fig. 4B, the PieR binding to the upstream of *pieR* and generated significantly shifted bands. DNase I footprinting assay with FAM-labeled primers uncovered one protected region (Fig. 4C) and the binding site is 5′-GGCCGCGACGGAGGAGGACGTCC GGGCGGGACCGGAGTGCCGGATG-TGCA-3' (Fig. 4D). The long protected sequence suggested that DNA secondary structure is important for PieR binding. The sequence analysis revealed two direct repeats 5′-CCGGA-3′ within this binding site (Fig. 4D). According to the typical regulatory mechanisms [23], it was proposed that PieR might directly up-regulate the transcription of the *pie* operon by acting on the identified binding sites in this region. Therefore, it can conclude that PieR binds to the upstream region of *pie* cluster and activates the whole gene cluster including itself, resulting in the translation of structural gene and post-modification gene simultaneously. Direct repeats within the target binding region seem to be a common characteristic of SARPs [38]. To explore the relationship of PieR with other SARPs, phylogenetic analysis was conducted. Based on the analysis it can be seen that PieR formed into the same clade with SARP protein AurD and was also close related to other two SARP positive regulators PolR [35] and SanG (Fig. 5).

4. Discussion

Streptomyces are well-known producers of tremendous biological active compounds, and the production of these antibiotics is usually controlled by multiple regulatory proteins, strictly coordinating with their growth and environmental conditions [39]. Transcriptional control that posits the genetic information flow commences with transcription, and accordingly, regulatory tools targeting transcription have received the most attention in terms of tool development and engineering applications. Typically, regulatory proteins act either through pleiotropic or via pathway specific mechanism to control the expression of individual antibiotic gene clusters [40]. Many pathway-specific regulatory proteins belong to SARPs family [34,35], and these proteins were proposed to bind to a direct repeat sequence overlapping with the −35 region of the target promoter [40]. The representative structure of SARPs contains the N-terminal OmpR-like DNA-binding domain, which is in charge of binding with repeated motif and a C-terminal transcriptional activation domain responsible for actuating the transcription of structural gene [41], while some proteins also contain a central ATPase domain with a potential ATP-binding motif [42,43].

Recently, structural analysis has contributed to the revealing of the regulatory mechanism of SARPs [41,44]. Especially, the study of AfsR in *Streptomyces coelicolor* A3 (2) provided a model for transcriptional activation by SARPs [44]. The AfsR protein acts with DNA and RNA polymerase in a dimer formation to form a complex binding to the −10 promoter region. The ATPase activity is essential for the isomerization of the closed complex between AfsR and RNA polymerase to a

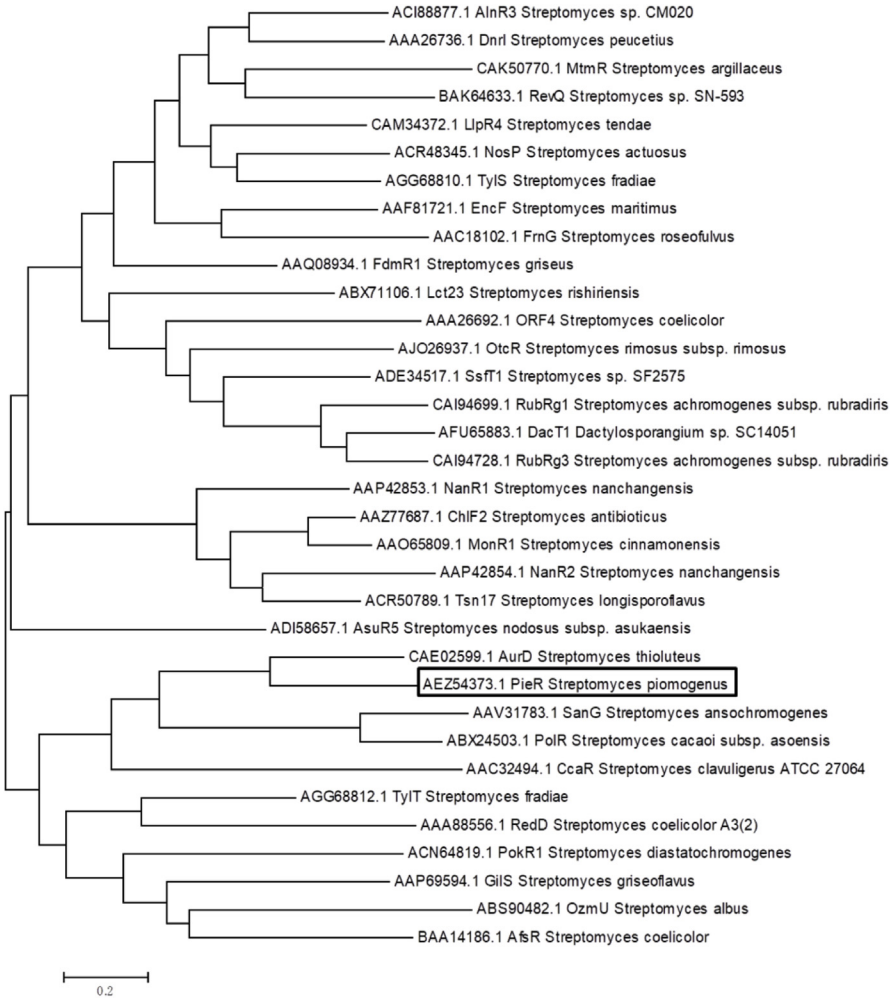

Fig. 5. Phylogenetic analysis of PieR with other SARP family proteins.

transcriptionally competent open complex. However, the regulatory mechanism of other SARP without ATPase, such as the recruitment of RNA polymerase for transcriptional activation still remains elusive.

The biosynthetic gene cluster of piericidin A1 only contains one possible regulatory gene *pieR*. The bioinformatics analysis of PieR suggested that it is a possible SARP family regulatory protein, containing N terminal conserved DNA binding HTH domain and C terminal BTAD domain, which are characteristic of SARP family proteins. Furthermore, PieR can be grouped into a clade designed "small SARPs" according to the number of amino acids (less than 300 amino acids long) [41]. In this study, the annotated SARP protein-encoding gene was manipulated genetically firstly, and was proved to play potent positive regulatory role in piericidin A1 biosynthesis. Consistently, the over-expression of *pieR* contributed to the higher yield of piericidin A1. Furthermore, the RT-PCR analysis showed that *pieR* directly regulated the transcription of all the genes that are organized into one operon. This regulation is concise and facilitates the coordinated expression of the biosynthetic genes to form effective biosynthetic machinery. The identified direct repeats in the possible binding region of PieR through EMSA and DNase I footprinting assay is consistent with the reported binding characteristic of SARP. The phylogenetic analysis of PieR with other SARPs showed that it formed into the same clade with other homologous "small SARP" AurD, while other homologous proteins SanG, NosP, TylT, AfsR spread on different clades. Nevertheless, it was also close related to two "large SARPs" PolR and SanG (Fig. 5). To date, both of pathway-specific and pleiotropic global SARPs have been reported to regulate the biosynthesis of natural products. Most of the SARPs act as activators to positively regulate the transcription of antibiotic gene clusters by binding specific sequence of target promoter, few has also been reported negatively control the biosynthesis, such as FarR4 [45]. Various regulatory modes have been reported, and the regulatory mechanism is strictly dependent on the domain arrangement. PolR and SanG have been reported to positively regulated the biosynthesis of polyoxin in *Streptomyces cacaoi* subsp. Asoensis [35] and nikkomycin in *Streptomyces ansochromogenes* [46], respectively. The two SARP proteins SanG and PolR possess three major functional domains: an OmpR-like DNA-binding domain, a central ATP-binding motif and a C-terminal half homologous to the guanylate cyclase domain of the LuxR family. The binded ATP was proposed to stabilize the confirmation of SanG, meanwhile, the ATP hydrolysis activity might account for the inactivation of the target gene. While AfsR, containing a motif similar to the characterized Walker-box ATPases and a C-terminal tetratricopeptide repeat domain, was proved to bind to the promoter region of regulatory gene. However, something is always exceptional. Recently, the "small SARP" NosP has been characterized to activate the nosiheptide production responding to both peptidyl and small-molecule ligands, which is unprecedent in Streptomyces. Considering the diverse regulatory mode, further studies focusing on the mechanism of PieR would facilitate the understanding of SARPs and on this basis set stage for developing potent regulatory elements toward effective synthetic route for valuable intermediates in response to pharmaceutical development.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2018.12.002.

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