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

Yan Li¹, Lingxin Kong¹, 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

**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 DNease 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, grazing 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 anti-inflammatory [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 fibroblast promotion 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

---

Peer review under responsibility of KeAi Communications Co., Ltd.

*Corresponding author.

E-mail address: dlyou@sjtu.edu.cn (D. You).

¹These authors contributed equally to this work.

https://doi.org/10.1016/j.synbio.2018.12.002

Received 24 November 2018; Received in revised form 5 December 2018; Accepted 5 December 2018

2405-805X/ © 2019 Production and hosting by Elsevier B.V. on behalf of KeAi Communications Co., Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/BY-NC-ND/4.0/).
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 pionmogaus* 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 *Streptomyces* 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 derivatives, the regulatory role of PieR from *Streptomyces pionmogaus* 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. pionmogaus* 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)/ployE was used as host for protein expression. pET28a (Novagen) was used as protein expression vectors. pLJ778 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 pLJ778 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. pionmogaus* var. Hangzhouwanensis by intergeneric conjugation. The double-crossover strain pieRΔpieR 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 pieRΔpieR 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 (H2O) and 20% solvent B (acetoniitrile) 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 First 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 Ndel and EcoR I, pieR was cloned into pET-
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 strain was cultured at 37 °C and 220 rpm in LB medium supplemented with kanamycin (final concentration is 50 g/ml) to OD600 = 0.6. Isopropylthio-β-D-galactoside (IPTG) with final concentration of 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 (1 M 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 MgCl2, 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 buffer 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 used.

Table 2

| Strains or plasmid | Description | Source |
|--------------------|-------------|--------|
| 5. promogues var. Hangzhouwuanensis | Wild-type strain producing pieridicin A1 | Zhejiang Academy of Agricultural Sciences |
| ΔpieR | a disruption of pieR by PCR targeting | This study |
| ΔpieR:pET28a | pieR gene of S. promogues var. Hangzhouwuanensis in pET28a | This study |
| ΔpieR:pSET152 | control strain of ΔpieR:pET28a | This study |
| OpieR | WT::pSET152-pieR, over-expression strain | This study |
| WT::pSET152 | control strain of OpieR | This study |

Table 1

| Strains or plasmids | Description | Source |
|---------------------|-------------|--------|
| pET28a | Protein expression vector | Novagen |
| pET28a derivative carrying pieR | E. coli strain BL21 (DE3) for PieR expression | Stratagene |
| pSET152 | Integrative plasmid used for complementation | Current study |
| pieR | Fosmid7D9 derivative which was partially replaced by spectinomycin resistance gene | This study |
| WT::pSET152 | Control strain of ΔpieR::pSET152 | This study |
| ΔpieR::pSET152 | Complementation strain | This study |
| ΔpieR | -C | This study |

Table 3

| Primer name | Sequence (5’-3’) |
|-------------|------------------|
| VLY-1R      | GGGGAAAGCTTGGAGAAGAGGAGACTAGT |
| VLY-1F      | CGGCGGTTTCTTCTGCTCGT |
| 16S-R       | CATGCAATGCTAAGCTGCAAGGTA |
| 16S-F       | GGGCGTGTCTAGAGTCCAGTG |
| PieR-R      | CGCGGTCTGCGCAACACCAT |
| PieR-F      | ACCAGAGCCGCGCTTCATCAG |
| RA1-R       | GGGGCTTCTAGGTCATCAGT |
| RA1-F       | AGGGTGGGTTCGCCTCTCC |
| A2A-2R      | AGGGGAGAGGAGCGAGAGAAGC |
| A2A-2F      | GGGGTTCGCGCCGAGAAGC |
| A2A3-R      | GAGAGCCGTGCTGCGACATG |
| A2A3-F      | ATGGTGACCGCGTTGAGGCTT |
| A3A4-R      | AGGAGCTCCTGGAGAGCTCAGT |
| A3A4-F      | TTCTGTGCGCGAGTGCAGGT |
| A4A5-R      | GTCGACGAGCTGCGGTGCTG |
| A4A5-F      | AGGAGACGAGGCGGAAGAAG |
| A681-R      | GGGCGAGAGCGAGCGAGAG |
| A681-F      | TGGGTGCGGTTGATGACG |
| B1C-R       | GGGGCCGCCGCGAGCCAGCG |
| B1C-F       | TGAGCTGACGAGCTCAGGC |
| CD-R        | CCTGCGAAGTCTTCTTCTC |
| CD-F        | GGTGTTGTGATGCGGTCAGT |
| DB2-R       | GACCTCTACGGCGAGACAT |
| DB2-F       | TTGGTCACAGGCTGCGTGAAG |
| B2E-R       | CGGCGGTTTGCTAGCGAGGAGG |
| B2E-F       | TCCGGTGGTACGAGCGAGG |
| pieR-R      | AAAAGAATTCCTAGGCGGCGAGCCAGGG |
| pieR-F      | AAAACAGATGTTGGTAGGTTGCTGTTGCGG |

Probes used in this study.

| Primer name | Sequence (5’-3’) |
|-------------|------------------|
| Probe1-F   | CGCCAGGGTTTTTCCCGGCAGCAGGAGGAGGAGGAGG |
| Probe1-R   | GGTTGTCAGCGAGCGAGGAGGAGGAGGAGGAGG |
| M13F-47    | CGCCAGGGTTTTTCCCGGCAGCAGGAGGAGGAGG |

Probes used in this study.
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 CaCl₂ 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 philantlii bv. triangulum (ARV85759.1); AurD, from Streptomyces thioluteus (ACE02599.1); TyIT, from Streptomyces fradiae (AGG68812.1); AfsR, from Streptomyces coelicolor (BA14186.1); SanG, from Streptomyces ainosochromogenes (AAV31783.1); NosP, from Streptomyces actuousus (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 philantlii 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-helices 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-helices (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 over-expressing strain (OpieR). After excluding the productivity change exerted by empty plasmid, the piericidin A1 production in OpieR 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 OpieR 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.
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 a His6-PieR recombinant protein (the full-length PieR protein) as a negative control. As a positive control, the gene was expressed in E. coli BL21 (DE3), an obvious overexpressed protein band (31 kDa) could be detected in the soluble component. Finally, the expressed pieR gene. This newly cloned fragment (774 bp) began with GTG and was then cloned into the pET28a plasmid for the construction of a His6-PieR recombinant protein (the full-length PieR protein) as a negative control. As a positive control, the gene was expressed in E. coli BL21 (DE3), an obvious overexpressed protein band (31 kDa) could be detected in the soluble component. Finally, the expressed pieR gene. This newly cloned fragment (774 bp) began with GTG and was then cloned into the pET28a plasmid for the construction of a His6-PieR recombinant protein (the full-length PieR protein) as a negative control. As a positive control, the gene was expressed in E. coli BL21 (DE3), an obvious overexpressed protein band (31 kDa) could be detected in the soluble component. Finally, the expressed
was incubated with purified PieR of gradient concentrations in a typical reaction system, which was composed of 100 mM KCl, 2.5 mM MgCl₂, 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′-GGCGCGGAACGGAGGTTCG-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).

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 SARP 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 SARP 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.](image-url)
transcriptionally competent open complex. However, the regulatory mechanism of other SARP without APase, 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 designated “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, TyiT, AfsR spread on different clades. Nevertheless, it was also close related to two “large SARPs” PoR 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 FarR [45]. Various regulatory modes have been reported, and the regulatory mechanism is strictly dependent on the domain arrangement. PoR and SanG have been reported to positively regulate the biosynthesis of polyoxin in Streptomyces coelicolor, used to regulate the activity of antibiotic gene clusters. The two SARP proteins SanG and PoR 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 bound 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 tetracistructype 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 unprecedented 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.

Funding

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; Shanghai Pujiang Program from the Shanghai Municipal Council of Science and Technology (12PJJD021); and China Postdoctoral Science Foundation (2017M620151).

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.

References

[1] Newman DJ, Cragg GM. Natural products as sources of new drugs from 1981 to 2014. J Nat Prod 2016;79:629–61.
[2] Cantrell CL, Dayan FE, Duke SO. Natural products as sources for new antibiotics. J Nat Prod 2012;75:1231–42.
[3] Hwang KS, Kim HJ, Charuasunti P, Palsson BO, Lee SY. Systems biology and bio-technology of Streptomyces species for the production of secondary metabolites. Biotechnol Adv 2014;32:255–68.
[4] Tuson RV. XXII. —note on an alkaloid contained in the seeds of the Ricasinum com- munis, or castor-oil plant. J Chem Soc 1864:17:195–7.
[5] Isaka M, Tanticharoen M, Kongsaeree P, Thettharanon Y. Structures of cordyppy-ridones A-D, antimalarial N-hydroxy- and N-methoxy-2-pyridones from the insect pathogenic fungus Cordyces pineae. J Org Chem 2001:66:4803–8.
[6] Dickinson JM, Hanson JR, Hitchcock PB, Claydon N. Structure and biosynthesis of harzianropyridone, an antifungal metabolite of Trichoderma harzianum. J Chem Soc Perkin Trans 1989:1:1885–7.
[7] Gui C, Li Q, Mo X, Qin X, Ma J, J. Discovery of a new family of Dieckmann cyclases essential to tetramic acid and pyridone-based natural products biosynthes- is. Org Lett 2015:17:628–31.
[8] Takahashi N, Suzuki A, Tamura S. Structure of piericidin A. J Am Chem Soc 1985;107:2966–4.
[9] Kroiss J, Kaltenpich M, Schneider B, Schwinger M, Hertweck C, Malduda R, et al. Symbiotic Streptomyces provide antibiotic combination prophylaxis for wasp offspring. Nat Chem Biol 2010;6:261–3.
[10] Shinguma RA, Helmeke E, Kelter G, Fiebig GB, Haast E, Schulz C, Liese C. Pyridocin P: a cy- tototic piericidin glucoside antibiotic produced by a marine Streptomyces isolate. J Antibiot 2016;64:205–9.
[11] Gutman M, Singer T, Reiner H, Casida J. 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, TyiT, AfsR spread on different clades. Nevertheless, it was also close related to two “large SARPs” PoR 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 FarR [45]. Various regulatory modes have been reported, and the regulatory mechanism is strictly dependent on the domain arrangement. PoR and SanG have been reported to positively regulate the biosynthesis of polyoxin in Streptomyces coelicolor, used to regulate the activity of antibiotic gene clusters. The two SARP proteins SanG and PoR 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 bound 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 tetracistructype 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 unprecedented 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.

Funding

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; Shanghai Pujiang Program from the Shanghai Municipal Council of Science and Technology (12PJJD021); and China Postdoctoral Science Foundation (2017M620151).
Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA. Practical Streptomyces genetics. Norwich, UK: John Innes Foundation; 2000.

Wang Y, Cen XF, Zhao GP, Wang J. Characterization of a new GlnR binding box in the promoter of amtB in Streptomyces coelicolor inferred a PhoP/GlnR competitive binding mechanism for transcriptional regulation of amtB. J Bacteriol 2012;194:5237–44.

Thompson JD, Higgins D, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22:4673–80.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011;28:2731–9.

Li J, Li Y, Niu G, Guo H, Qiu Y, Lin Z, et al. NosP-regulated nosiheptide production responds to both peptidyl and small-molecule ligands derived from the precursor peptide. Cell Chem Biol 2018;25:143–153 e4.

Li R, Xie Z, He X, Chen W, Deng Z, et al. PolY, a transcriptional regulator with ATPase activity, directly activates transcription of polR in polyoxin biosynthesis in Streptomyces cacaoi subsp. asoensis. Microbiology 2009;155:1819–31.

Anton N, Mendes M, Martin J, Aparicio JF. Identification of PimR as a positive regulator of pimaricin biosynthesis in Streptomyces natalensis. J Bacteriol 2004;186:2567–75.

Tanaka A, Takano Y, Ohnishi Y, Horinouchi S. AfsR recruits RNA polymerase to the afsS promoter: a model for transcriptional activation by SARPs. J Mol Biol 2007;369:322–33.

Kurniawan YN, Kitani S, Maeda A, Niiro T. Differential contributions of two SARP family regulatory genes to indigoidine biosynthesis in Streptomyces lavendulae FRI-5. Appl Microbiol Biotechnol 2014;98:9713–21.

He X, Li R, Pan Y, Liu G, Tan H. SanR, a transcriptional activator, controls nikkomycin biosynthesis through binding to the sanN-sanO intergenic region in Streptomyces anschromogenes. Microbiology 2010;156:828–37.

Bierman M, Logan R, O’Reien K, Seno ET, Rao RN, Schoner BE. Plasmid cloning vectors for the conjugal transfer of DNA from Escherichia coli to Streptomyces spp. Gene 1992;116:43–9.