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Global and Pathway-specific Transcriptional Regulations of Pactamycin Biosynthesis in *Streptomyces pactum*

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Abstract

Pactamycin, a structurally unique aminocyclitol natural product isolated from *Streptomyces pactum*, has potent antibacterial, anti-tumor and anti-protozoa activities. However, its production yields under currently used culture conditions are generally low. To understand how pactamycin biosynthesis is regulated and explore the possibility of improving pactamycin production in *S. pactum*, we investigated the transcription regulations of pactamycin biosynthesis. *In vivo* inactivation of two putative pathway-specific regulatory genes, *ptmE* and *ptmF*, resulted in mutant strains that are not able to produce pactamycin. Genetic complementation using a cassette containing *ptmE* and *ptmF* integrated into the *S. pactum* chromosome rescued the production of pactamycin. Transcriptional analysis of the *ptmE* and *ptmF* strains suggests that both genes control the expression of the whole pactamycin biosynthetic gene cluster. However, attempts to overexpress these regulatory genes by introducing a second copy of the genes in *S. pactum* did not improve the production yield of pactamycin. We discovered that pactamycin biosynthesis is sensitive to phosphate regulation. Concentration of inorganic phosphate higher than 2 mM abolished both the transcription of the biosynthetic genes and the production of the antibiotic. Draft genome sequencing of *S. pactum* and bioinformatics studies revealed the existence of global regulatory genes, e.g., genes that encode a two-component PhoR-PhoP system, which are commonly involved in secondary metabolism. Inactivation of *phoP* did not show any significant effect to pactamycin production. However, in the *phoP::aac(3)IV* mutant, pactamycin biosynthesis is not affected by external inorganic phosphate concentration.

Keywords

pactamycin; transcriptional regulation; biosynthesis; genetic engineering

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INTRODUCTION

The Gram-positive, filamentous, soil bacteria *Streptomyces* are notable for their ability to produce a wide variety of secondary metabolites with interesting pharmacological activities. The actinomycetes, the family to which the streptomycetes belong, are attributed to the production of over two-thirds of known antibiotics (Berdy 2005). Antibiotic production in streptomycetes exhibits in a growth-phase-dependent manner, which is controlled by both global and pathway-specific regulatory genes whose expression frequently depends on physicochemical factors including nutrient supply, cell density, oxygenation, temperature and pH (Bibb 1996).

A number of regulatory systems with pleiotropic effects on antibiotic production in streptomycetes have been identified and studied extensively (Bibb 1996, Martin and Liras 2010, Martin et al. 2011, Liu et al. 2013). Bacterial hormones, γ -butyrolactones, are active at nanomolar concentrations and induce expression of multiple biosynthetic gene clusters by modulating the DNA binding activity of cognate receptors (Horinouchi 2002, Takano 2006). Another example is the two-component PhoR-PhoP system, which is well studied for regulation of antibiotic biosynthesis induced by phosphate starvation (Martin and Liras 2010, Martin et al. 2011). The genes required for assembly of secondary metabolites in *Streptomyces* are usually clustered together with pathway-specific regulatory genes that control the onset of biosynthesis (Bibb and Hesketh 2009). Numerous pathway-specific regulators have been identified in *Streptomyces* (e.g., ActII-ORF4, RedD, CdaR), many of which belong to the *Streptomyces* antibiotic regulatory protein (SARP)-family proteins with a typical OmpR-like conserved domain in the N-terminus that specifically regulate the transcription of certain biosynthetic genes (Wietzorrek and Bibb 1997, Sheldon et al. 2002). Analysis and proper manipulations of these regulatory systems is crucial for understanding the mechanism of regulation and rationally enhance secondary metabolite production (Lu et al. 2017).

Pactamycin (Fig. 1a), a cyclopentitol-containing protein synthesis inhibitor, is produced by the soil bacterium *Streptomyces pactum* (Bhuyan 1962). This antibiotic has been known to have broad-spectrum growth inhibitory activity against bacteria (Bhuyan 1962), mammalian cells (White 1962), viruses (Taber et al. 1971), and protozoa (Otoguro et al. 2010). Recently, through a biosynthetic engineering technology, a series of mutant strains of *S. pactum* ATCC 27456 have been generated, and many of them produce new analogues of pactamycin (Ito et al. 2009, Lu et al. 2011, Almabruk et al. 2013, Abugrain et al. 2016). To date, more than two dozen pactamycin analogues have been isolated from the wild-type and the mutant strains of *S. pactum* (Hara et al. 1964, Kondo et al. 1964, Wiley et al. 1970, Rinehart et al. 1980, Dobashi et al. 1986, Hurley et al. 1986, Ito et al. 2009, Lu et al. 2011, Iwatsuki et al. 2012, Abugrain et al. 2016, Abugrain et al. 2017). A number of them, e.g., TM-026 (Fig. 1a), showed superiority to the parent compound in terms of chemical stability and selectivity toward malarial parasites (Lu et al. 2011). However, the production yields of pactamycin (about 2–3 mg/L) from *S. pactum* ATCC 27456 and its congeners are relatively low, hampering both mechanistic and preclinical studies of these compounds. Therefore, we set out to investigate the transcriptional regulation of pactamycin biosynthesis, as the knowledge

of which may enable the creation of new strains of *S. pactum* with higher pactamycin production capability.

Previously, we have identified the biosynthetic gene cluster of pactamycin within an 86-kb sequenced region of DNA from *S. pactum* ATCC 27456 (Ito et al. 2009). Armed with the tools needed to genetically disrupt target genes in *S. pactum*, we were able to determine the boundaries and identify a number of putative regulatory genes within the cluster. Disruptions of genes upstream of *ptmZ* and downstream of *ptmW* (Fig. 1b) gave no phenotypic differences, compared to the wild-type (data not shown), suggesting they are not involved in pactamycin biosynthesis. Based on these gene inactivation experiments and bioinformatics analysis, we predict that the pactamycin gene cluster is located between *ptmZ* and *ptmW* (Fig. 1b).

Within the pactamycin biosynthetic gene cluster, there is a gene (*ptmF*) that encodes a putative transcription regulatory protein, which contains DNA-binding winged helix-turn-helix (HTH) domain. This protein belongs to the family of transcriptional regulatory protein C-terminal (Trans_reg_C). PtmF shows some similarity to DNA binding response regulators or two-component transcription regulators (Kenney 2002). Amino acid sequence alignment of PtmF with other response regulators revealed that it shares high similarity with OmpR-like motif of two-component regulators (Fig. S1). Thus, this protein may be involved in the transcriptional regulation of the pathway and was selected as a target for gene inactivation. Right downstream of *ptmF* is a gene (*ptmE*) that codes for a hypothetical protein. The deduced product of *ptmE* belongs to a P-loop containing ATPase superfamily, which contains a nucleotide-binding motif (p-loop) at the N-terminus. While little is known about the role of such protein in secondary metabolism, there is a case where a transcriptional regulator contains an OmpR-like DNA binding domain in its N-terminal and an ATPase domain in the middle of the protein (Li et al. 2010). Therefore, we propose that the products of *ptmF* and *ptmE* may cooperatively control the biosynthesis of pactamycin.

In the present study, we investigated the role of *ptmE* and *ptmF* in pactamycin biosynthesis through *in vivo* gene inactivation, complementation experiments, and transcriptional analysis. In addition, we investigated the effects of phosphate starvation on pactamycin biosynthesis. Further, we carried out draft genome sequencing of *S. pactum* and identified genes that are involved in the two-component PhoR-PhoP system in *S. pactum*.

MATERIALS AND METHODS

Bacterial strains and plasmids

Pactamycin producing *S. pactum* ATCC 27456 was purchased from American Type Culture Collection (ATCC). *Escherichia coli* DH10B was used as a host strain for the construction of recombinant plasmids. *E. coli* ET12567/pUZ8002 was used as donor strain in conjugation experiments. pBlueScript II (SK-) (Stratagene) and pGEM-T Easy (Promega) were used as cloning vectors. pTMN002 is a pIJ101 derivative containing an oriT transfer element required for conjugation (Ito et al. 2009). pTMW050 is a pSET152 derivative containing *ermE** promoter (Lu et al. 2011). pIJ6902 is another pSET152 derivative containing *tipA*

promoter (Huang et al. 2005). Other bacterial strains and plasmids used in this study are listed in Table S1.

General DNA manipulations

Genomic DNA of *S. pactum* ATCC 27456 was prepared by standard protocol (Kieser et al. 2000) or using the DNeasy Tissue Kit (QIAGEN). DNA fragments were recovered from an agarose gel by using the QIAquick Gel Extraction Kit (QIAGEN). Restriction endonucleases were purchased from Invitrogen or Promega. Preparation of plasmid DNA was done by using a QIAprep Spin Miniprep Kit (QIAGEN). All other DNA manipulations were performed according to standard protocols (Kieser et al. 2000, Sambrook and Russell 2001). PCR was performed in 30 cycles by using a Mastercycler gradient thermocycler (Eppendorf) and Platinum *Taq* DNA polymerase (Invitrogen) or Platinum *Pfx* DNA polymerase (Invitrogen). Oligodeoxyribonucleotides for PCR primers were synthesized by Sigma-Genosys, and are shown in Table S2. The nucleotide sequences of the gene fragments were determined at the Center for Genome Research and Biocomputing (CGRB) Core Laboratories, Oregon State University.

Construction of *ptmF* and *ptmE* mutants

Two ~1 kb PCR fragments upstream (*Hind*III/*Eco*RI) and downstream (*Eco*RI/*Xba*I) of the *ptmF* and *ptmE* genes were fused and cloned into the *Hind*III/*Xba*I site of pBluescript II SK(–) vector. The products were excised and cloned into the *Hind*III/*Xba*I site of pTMN002 to create pTMW067 and pTMW068, respectively. All plasmids were then individually introduced into *S. pactum* ATCC 27456 by conjugation using the *E. coli* donor strain ET12567/pUZ8002. Apramycin resistant strains representing single crossover mutants were obtained and subsequently grown on nonselective mannitol-soy flour agar containing 10 mM magnesium chloride (MS-Mg) to induce the formation of double crossover recombinants. Apramycin sensitive colonies were counter-selected by replica plating on MS-Mg agar with and without apramycin (50 µg/mL). The resulting double-crossover candidate strains were confirmed by PCR amplification with external primers flanking the respective targeted gene (Table S2). The resulting PCR fragment from putative double crossover mutants was subcloned into the pGEM-T vector and sequenced to confirm that part of the gene has been removed from the chromosome.

Genetic complementation with *ptmF*, *ptmE*, and *ptmG-ptmF-ptmE* genes

The *ptmF*, *ptmE*, *ptmF-ptmE*, and *ptmG-ptmF-ptmE* genes were amplified by PCR and cloned into the *Eco*RV site of pBluescript II SK(–) vector. The products were then excised and cloned into the *Nde*I/*Xba*I site (for *ptmF*, *ptmE*, and *ptmF-ptmE*) or the *Eco*RI/*Xba*I site (for *ptmG-ptmF-ptmE*) of pTMW050 to create pTMW069, pTMW070, pTMW071, and pTMM046, respectively. pTMW069, pTMW070, and pTMM046 were then individually introduced into the corresponding *ptmF* and *ptmE* mutants. Genetic complementation strains were obtained based on apramycin resistance and confirmed by PCR.

Gene duplication in *S. pactum*

The *ptmF*, *ptmE* and *ptmF-ptmE* genes were excised from pTMW069, pTMW070, and pTMW071 and cloned into the *NdeI/XbaI* site of pIJ6902 to create pTMW072, pTMW073 and pTMW074, respectively. The plasmids pTMW069, pTMW070, pTMW071, pTMW072, pTMW073 and pTMW074 were then individually introduced into *S. pactum* to generate over-producing strains.

ptmG-ptmF-ptmE operon duplication in *S. pactum* *ptmH* mutant

The *ptmG-ptmF-ptmE* fragment was excised from pTMM046 and cloned into the *NdeI/XbaI* site of pIJ6902 to create pTMZ002. pTMZ002 plasmid was introduced into *S. pactum* *ptmH* mutant to generate over-producing strains.

RNA isolation and reversed-transcription PCR (RT-PCR)

Wild-type and mutant strains of *S. pactum* were inoculated into production medium and cells were harvested for RNA extraction after 1–6 days incubation. RNAs were extracted from the cells using Trizol (Invitrogen) after a 30 min treatment of the cells with lysozyme. RNAs were precipitated by an equal volume of isopropanol and washed with 70% ethanol. The RNA samples were then treated with DNase (RNase-free, Invitrogen) to remove residual genomic DNA. After quantification, RNA (1 µg) was reverse-transcribed into cDNA (20 µL) using random primers and SuperScript II reverse transcriptase (Invitrogen). cDNA products (0.5 µL) were then subjected to PCR amplification (25 cycles). RT-PCR primers are listed in Table S2. Primers for 16S rRNA gene were used as an internal control. PCR conditions were: template DNA denatured at 94 °C for 4 min, then 94 °C 30 sec, 60 °C 30 sec, 72 °C 30 sec, for 25 cycles.

Analysis of metabolites from *S. pactum* wild-type, *ptmF*, and *ptmE* mutants

S. pactum were grown on BTT agar [glucose (1%), yeast extract (0.1%), beef extract (0.1%), casein hydrolysate (0.2%), agar (1.5%), pH 7.4] at 30 °C for 3 days. Single colonies were used to inoculate the BTT seed cultures and incubated at 30 °C for 2 days. Production cultures were prepared in modified Bennett medium (Sakuda et al. 2001) (50 mL) and inoculated with seed cultures [10% (V/V)]. Apramycin (5 µg/mL) was added to cultures of strains that harbor pTMW050 derived plasmids. Thiostrepton (5 µg/mL) was added to induce *tipA* promoter. The production cultures were incubated at 30 °C for 6 days under vigorous shaking (200 rpm). The mycelia were centrifuged and the supernatants were extracted twice with equal volumes of EtOAc. The organic solvent was evaporated *in vacuo* and the residues were dissolved in MeOH (1.8 mL) and analyzed by reverse-phase HPLC or LC-MS. Analysis of the metabolites of the *S. pactum* was carried out by reverse-phase HPLC with a C-18 silica gel column (YMC- ODS A 5 µ, 4.6 × 250 mm, flow rate 0.30 mL/min for quantification the production yield or 0.40 mL/min for comparison of *S. pactum* growth in different phosphate concentration) with the following solvent gradient program [solvent A (5 mM NH₄OAc solution) and B (MeOH–MeCN = 4:1)] was used: 0–45 min, % B = 60; 45–48 min, % B = 60–98; 48–60 min, % B = 98; 60–63 min, % B = 98–60; and 63–80 min, % B = 60. Peaks eluting from the column were monitored at 240 nm. The molecular mass of each compound was determined by electrospray mass spectrometry. For

quantification of pactamycin analogues in the wild-type and the mutant strains, standard curves and equation were generated according to the correlation between areas under the curves of HPLC peaks and injection amount of authentic pactamycin analogues (Fig. S2). Experiments were performed in triplicate. The yields of pactamycin analogues produced by the wild-type and the mutant strains were calculated accordingly.

Growth measurement of *S. pactum*

We used a protein extraction protocol to measure cell growth over time. Cell treatment procedures were modified from a previous report (Meyers et al. 1998). *S. pactum* cell cultures (10 mL) were centrifuged at 5,000 rpm for 15 min. The pellets were washed with phosphate-buffered saline (PBS) pH 7.0 (1 mL) (without resuspending the cells), and were centrifuged at 14,000 rpm for 5 min. The pellets were resuspended in 1 M NaOH (0.2 mL) and the sealed tubes were placed in boiling water for 10 min. The samples were neutralized by adding 5 M HCl (0.04 mL), and the volumes were adjusted to 1 mL by adding PBS pH 7.0 (0.76 mL). Samples were centrifuged for 30 min, and 0.8 mL of each supernatant was used for protein determination. The samples were measured at 230 nm and 260 nm using a Nanodrop 1000, and the protein concentration was calculated using the equation: Total protein ($\mu\text{g/mL}$) = $(183 \times A_{230}) - (75.8 \times A_{260})$. The dilution factor is 10.

Sequencing of *S. pactum* genome

The draft sequence of *S. pactum* genome was determined using a Genome Analyzer II (Illumina). Approximately 5.0 μg of chromosomal DNA was used for library construction and sequencing, according to the procedure recommended by Illumina Inc. DNA assembly was performed by the CLC genomic workbench software (CLC Bio) and the tagcleaner.pl perl script to give 4,569 contigs (total 7,457,333 bp). The nucleotide sequences of the pactamycin biosynthetic gene cluster and the *phoP* gene have been deposited in GenBank accession numbers FJ392609 and MH800192, respectively.

Inactivation of the pleiotropic regulatory gene *phoP*

A 920-bp internal fragment of *phoP* was generated by PCR using primers *phoP-F/phoP-R* and *S. pactum* genomic DNA as template. The PCR product was cloned into the *HindIII/XbaI* sites of pTMM002 to create pTMM037. The correct construct was verified by PCR and DNA sequencing. Plasmid pTMM037 was then introduced into *S. pactum ptmH* mutant by intergeneric conjugation using the *E. coli* donor strain ET12567/pUZ8002 according to the method described in our previous publication (Lu et al. 2011). The disruption of *phoP* was confirmed by PCR amplification (Fig. S3).

Quantitative analysis of TM-026 production in *ptmH/phoP::aac(3)IV* mutant

S. pactum ptmH/phoP::aac(3)IV was grown in modified Bennett medium (50 mL) containing apramycin (5 $\mu\text{g/mL}$) as described above. The culture was incubated at 30 °C for 6 days under vigorous shaking (200 rpm). The mycelia were centrifuged and the supernatants were extracted twice with equal volumes of EtOAc. The organic solvent was evaporated *in vacuo* and the residues were dissolved in MeOH (2 mL) and analyzed by reverse-phase HPLC using a C-18 silica gel column. The following solvent gradient

program, solvent A (0.1% (v/v) TFA aqueous solution) and B (0.1% (v/v) TFA in MeOH), was used: 0–40 min, % B = 40; 40–50 min, % B = 100; 50–54 min, % B = 40. Peaks eluting from the column were monitored at 254 nm. To study the effect of *phoP* inactivation on the production of TM-026 production in *S. pactum ptmH*, a standard curve and equation were generated according to the correlation between areas under the curves of HPLC peaks and injection amount of authentic TM-026.

Effect of inorganic phosphate on pactamycin biosynthesis in *S. pactum ptmH* and *ptmH/phoP::aac(3)IV*

S. pactum ptmH and *ptmH/phoP::aac(3)IV* were grown in modified Bennett medium (25 mL) containing different inorganic phosphate concentrations (0.25 mM – 10 mM). The production cultures were incubated at 30 °C for 6 days under vigorous shaking (200 rpm). The cultures were centrifuged and the supernatants were separated and extracted twice with equal volumes of EtOAc. The organic solvent was evaporated *in vacuo* and the residues were dissolved in MeOH (1.8 mL) and analyzed by reverse-phase HPLC with a C-18 silica gel column (Zorbax SB 5 μ , 4.6 $\times$ 150 mm) with a flow rate of 0.40 mL/min. The following solvent gradient program, solvent A (0.1% (v/v) TFA aqueous solution) and B (0.1% (v/v) TFA in MeOH), was used: 0–40 min, % B = 40; 40–50 min, % B = 100; 50–54 min, % B = 40. Peaks eluting from the column were monitored at 254 nm.

Antibacterial activity assay

Antibacterial activity of pactamycin and its analogues was determined by agar diffusion assay. *Staphylococcus aureus* was streaked on nutrient agar (Difco) and grown overnight at 37 °C. Colonies were transferred to nutrient broth and incubated at 37 °C for 24 h. Turbidity of the inoculum was measured to a proper density at 600 nm (BioRad, SmartSpec 3000). For plate preparation, inoculum (500 μ L) was mixed thoroughly with warm nutrient agar (50 mL) and poured to 25 mL square plates. The agar plates were allowed to solidify and dry for 30 min before assay. Sterile blank paper disks (Becton-Dickinson) were impregnated with MeOH solutions of crude extract of *S. pactum* at various concentrations (5 μ L) and dried at room temperature. The disks were placed onto inoculated agar plates and incubated at 37 °C for 24 h. In order to produce a contrast background of the inhibition zone, 0.25% MTT developing dye (1 mL) was added over the plates.

RESULTS

Genetic analysis of the putative regulatory genes in the pactamycin biosynthetic gene cluster

Bioinformatics analysis of the pactamycin biosynthetic gene cluster indicated that the putative response regulator PtmF and the putative ATPase containing PtmE may cooperatively control the biosynthesis of pactamycin. To determine the involvement of these putative regulatory genes in pactamycin biosynthesis, we inactivated the genes individually. Targeted in-frame deletion of *ptmF* was achieved by cloning upstream and downstream flanking regions of *ptmF* into pTMN002, transferring the resulting plasmid (pTMW067) into *S. pactum* via conjugation, and selecting for double crossover recombinants lacking the

ptmF gene sequence (Fig. 2a). The *ptmF* mutant was confirmed by PCR amplification (Fig. 2b) and DNA sequencing. The same approach was applied to generate *ptmE* mutant.

The effect of *ptmF* and *ptmE* inactivation on pactamycin production was determined by cultivation of the resulting mutants and analyzing their metabolic profiles by HPLC and LC-MS (Figs. 2c and 2d). The results showed that mutant strains lacking *ptmF* or *ptmE* were no longer able to produce pactamycin and its congeners, *e.g.*, pactamycate and 7-deoxypactamycin, suggesting that PtmF and PtmE are essential regulators, most likely involved in the transcriptional initiation of structural genes, or act as switches specific for pactamycin production.

Gene complementation with *ptmF*, *ptmE*, and the *ptmG-ptmF-ptmE* operon

To confirm that there is no polar effect involved in *ptmF* and *ptmE*, complementation experiments were performed by cloning *ptmF* or *ptmE* genes into the integration vector pTMW050, a pSET152 derivative containing the *aac3(IV)* gene and an *ermE** promoter, and then introducing into the corresponding mutants. The results showed that all conjugants were able to produce pactamycin, albeit in very low yields (data not shown). This partial rather than full restoration of pactamycin production may be due to the low efficiency of the *ermE** promoter in *S. pactum* strain or the lack of transcription initiation activity of the *ermE** promoter used in the constructs. Similar results have been observed in other *S. pactum* mutants as well (Lu et al. 2011).

Located upstream of *ptmF* is *ptmG*, which codes for a putative deacetylase. As *ptmG*, *ptmF*, and *ptmE* are present in a single operon (Fig. 1b), we decided to amplify and clone the three genes together, including a 100 bp sequence upstream of *ptmG* that harbors the native promoter and ribosomal binding site sequences, into the vector pTMW050. The resulting plasmid pTMM046 was then introduced into the *ptmF* and *ptmE* mutants and the phenotypes were analyzed by HPLC and ESI-MS. The results unambiguously showed restored production of pactamycin and its congeners (Fig. 3).

Transcriptional control of pactamycin biosynthetic genes

To better understand the timing of pactamycin gene expression, we compared the growths of the wild-type and the mutant strains of *S. pactum*, the production yields of pactamycin analogues, as well as the expressions of a set of genes representing operons in the cluster (Fig. 4). The growth of the wild-type and *ptmF* mutant strains of *S. pactum* were determined by a rapid and simple protein extraction protocol to measure cell growth over time. Compared to the wild-type strain, the *ptmF* mutant shows no differences in morphology and cell growth rate (Fig. 4a).

Pactamycin production in *S. pactum* occurs in a growth-phase-dependent manner. In the wild-type strain, the production of pactamycin and its congeners begin at Day 1, however, it becomes more pronounced during the late exponential growth stage (around 48 h), and ultimately reached the highest production at Day 5 (Fig. 4b). Antibacterial assay against *Staphylococcus aureus* also demonstrated that the production of the antibiotics increased from Day 1 to Day 3, whereas no antibiotic production was observed in the *ptmF* mutant (Fig. 4c).

To analyze the time course of the transcription of the pactamycin biosynthetic genes, we initially chose five genes and carried out RT-PCR analysis. Those genes include *ptmF* and *ptmE*, which are putative regulatory genes, and *ptmT*, *ptmC*, and *ptmH*, which are structural genes involved in different stages of pactamycin biosynthesis: 3-aminobenzoic acid formation, cyclopentitol ring formation, and tailoring process, respectively. The RT-PCR results revealed that observable transcription of pactamycin genes all starts at around 36 h (the late exponential stage), and continued to express until the cell death phase (Day 6). This somewhat contradicts with the detection of pactamycin congeners in Day 1, as showed in HPLC analysis and antibacterial assay. However, the antibiotic detected in Day 1 may be originated from seed cultures, as 10% (v/v) of the seed cultures were added to the production cultures. Nevertheless, based on the current data, samples collected at Day 3 (early stationary phase) were chosen for further transcriptional analysis.

The pactamycin biosynthetic gene cluster consists of multiple transcription units. The genetic organization of the cluster suggests the presence of at least six polycistronic transcriptional units (*ptmB-A*, *ptmC-D*, *ptmG-E*, *ptmH-N*, *ptmT-Q*, and *ptmU-V*) and two monocistronic units (*ptmO* and *ptmP*) (Fig. 1b). We selected at least one gene from each operon to analyze the effects of *ptmF* and *ptmE* disruption on pactamycin gene transcription.

Figure 5 shows comparative transcription profiles of *S. pactum* wild-type and *ptmF* and *ptmE* mutants at the early stationary stage. As our initial gene inactivation experiments and bioinformatics analysis suggest, there is only one response regulator in the gene cluster, PtmF, which works cooperatively with PtmE. RT-PCR result demonstrated a complete abolishment of structural gene expression when either *ptmF* or *ptmE* is disrupted. The transcription of *ptmE* and *ptmF* was also completely abolished. Therefore, it is concluded that the transcription of the whole gene cluster is controlled by the PtmF-PtmE system.

Duplication of the *ptmF*, *ptmE*, or *ptmF-ptmE* genes in *S. pactum*

The abolishment of pactamycin biosynthesis in the *ptmF* and *ptmE* mutants underscores the importance of these regulatory genes. Therefore, we explored the possibility of increasing pactamycin production by introducing a second copy of *ptmF*, *ptmE*, or *ptmF-ptmE* into *S. pactum*. The genes were cloned into the integrative vector pTMW050 (contains *ermE** promoter) or pIJ6902 (contains *tipA* promoter) to give pTMW069, pTMW070, pTMW071, pTMW072, pTMW073, pTMW074, respectively (Table S1). The plasmids were then introduced into *S. pactum* and the production of pactamycin and its congeners was analyzed quantitatively by HPLC. However, there were no significant differences in the antibiotic production between the engineered and the wild-type strains of *S. pactum* (data not shown).

Duplication of the *ptmG-ptmF-ptmE* operon in *S. pactum ptmH* mutant

As described above, genetic complementation with the *ptmG-ptmF-ptmE* operon provided a better result than those with *ptmF* or *ptmE* alone. Therefore, the *ptmG-ptmF-ptmE* operon was obtained from pTMM046 and ligated into pIJ6902. The resulting plasmid pTMZ002 was then introduced into *S. pactum ptmH*, which produces TM-026 – a pactamycin analogue. This strain was used because TM-026 is more stable than pactamycin, allowing more accurate quantification of the production yield. TM-026 production in *S. pactum*

ptmH and that containing a second copy of the *ptmG-ptmF-ptmE* operon was analyzed by HPLC. Unfortunately, similar to the results with *ptmF* or *ptmE* alone, duplication of the *ptmG-ptmF-ptmE* operon also did not show any significant improvement in the production yield of TM-026 (Fig. 6). While PtmE and PtmF are critical for gene transcription in pactamycin biosynthesis, there may be other mechanisms in *S. pactum* that play a role in balancing the homeostasis of the cell and controlling the production of pactamycin.

Effects of inorganic phosphate on the transcription of pactamycin biosynthetic genes

In addition to the investigation of the pathway-specific regulators, we also explored the effects of environmental signals, such as pH, temperature and nutrition factors, on the production of pactamycin. We found that the optimal pH for the production of pactamycin is between 7 and 8. Temperature does have effects on the overall production pattern of secondary metabolites by *S. pactum*. However, there are no significant differences in terms of pactamycin production yields between cultures at 30 °C and 37 °C. On the other hand, we found that the production of pactamycin is influenced by phosphate. Increasing concentrations of inorganic phosphate added to the cultures at inoculation time drastically reduced the production of pactamycin (Fig. 7a). When initial phosphate concentration is higher than 0.5 mM, the production yield of pactamycin decreased considerably. Almost no pactamycin was produced in cultures with the phosphate concentration more than 2 mM. Consistently, the RT-PCR results also show that the transcription of pactamycin genes was completely inhibited when the phosphate concentration is higher than 2 mM (Fig. 7b). The production of other secondary metabolites, such as NFAT-133 and piericidins also decreased in cultures with high phosphate concentrations. In the presence of lower concentrations of phosphate (<2 mM) significant amplification of the transcripts was observed. However, quantitative analyses of the products by HPLC indicate that the production yield is best when the concentration of inorganic phosphate is 0.25 mM or less.

Genome sequence of *S. pactum* and identification of global regulatory genes

The negative effect of inorganic phosphate to antibiotic production has been proposed to be mediated by the two-component PhoR-PhoP system (Martin and Liras 2010, Martin et al. 2011). To investigate the involvement of this system in pactamycin biosynthesis, we obtained a draft genome sequence of *S. pactum* and examined the presence of the two-component regulatory genes. Genome sequencing was accomplished using Illumina Genome Sequencer II and reads were assembled using Velvet to give 4,569 contigs. Overall genome size is 8.18 Mb with the GC content of 72.4%. The genome was annotated using BLASTX and CLC main workbench. We discovered within the genome the *phoU-phoR-phoP* cluster, which may be involved in regulating secondary metabolite production in *S. pactum*. The *phoP* gene encodes a protein that is an environmental phosphate response regulator (Sola-Landa et al. 2003). It is highly similar to the well-characterized *phoP* gene from *Streptomyces natalensis* (98% similarity) (Mendes et al. 2007). PhoP and PhoR form a two component regulatory system where PhoP is activated via phosphorylation mediated by the membrane bound PhoR (Fernandez-Martinez et al. 2012, Santos-Beneit 2015). Phosphorylated PhoP forms dimers, which act as a transcriptional activator or repressor (Liu and Hulet 1997). Through transcriptional activity, PhoP links phosphate uptake and metabolism with other nutrients. PhoP has very broad effects in *Streptomyces* spp., and the

disruption of *phoP* affects secondary metabolism differently in each species (Martin et al. 2017), making it an interesting target for gene disruption in *S. pactum*, especially since phosphate had an effect on pactamycin biosynthesis.

Inactivation of *phoP*

To study the potential effects of the two components PhoR-PhoP system on the production of pactamycin and its analogues, gene disruption of *phoP* was performed in TM-026 producer *S. pactum* *ptmH*. However, no significant effect on the production of TM-026 was observed in the *ptmH/phoP::aac(3)IV* mutant. Additionally, the production of TM-026 in *S. pactum* *ptmH/phoP::aac(3)IV* was tested in different inorganic phosphate concentrations to study the effect of PhoP component disruption on the sensing of inorganic phosphate in culture medium. Interestingly, unlike in *S. pactum* *ptmH*, the production of TM-026 in *S. pactum* *ptmH/phoP::aac(3)IV* was not significantly affected by different inorganic phosphate concentrations up to 10 mM (Fig. 8). In contrast, the production of piericidins was considerably decreased in cultures with 5mM and higher phosphate concentrations.

DISCUSSION

In *Streptomyces*, biosynthesis genes corresponding to a certain secondary metabolite are usually clustered together. Most clusters contain genes that encode pathway-specific regulatory proteins that control the transcription of the corresponding structural genes. In addition, global regulatory genes, typically outside the biosynthetic gene clusters, pleiotropically control the production of multiple metabolites. The regulatory network could be very complex, consisting both global and pathway-specific regulators. As part of our ongoing studies on the biosynthesis of the antitumor antibiotic pactamycin, we have investigated the regulatory network involved in the pathway with the end goal of developing genetically engineered strains that can produce more pactamycin. In the present study, we identified two pathway-specific regulatory genes, *ptmF* and *ptmE*, which play an essential role in transcriptional regulation of pactamycin biosynthesis.

Sequencing of the pactamycin biosynthetic gene cluster revealed the presence of *ptmF* gene, which contains a DNA-binding winged HTH domain and encodes a putative response regulator that belongs to the Trans_reg_C family. Phylogenetic analysis of PtmF indicates that it is closely related to DeoR transcriptional regulators, and shares high similarity with the OmpR-like DNA binding motif of two-component regulators, which is the characteristic of the SARP family. On the other hand, the deduced product of *ptmE* contains a nucleotide-binding motif (p-loop) at the N-terminal and is proposed to have kinase activity. We hypothesize that PtmF and PtmE work cooperatively to control the transcription of pactamycin biosynthetic genes. Indeed, gene inactivation of *ptmF* and *ptmE* resulted in mutant strains that were unable to produce pactamycin, suggesting that these two regulatory genes are positive regulators and essential for pactamycin biosynthesis.

To determine the effects of PtmF and PtmE on the transcription of pactamycin biosynthetic genes, we examined the expression of 13 genes (which cover all 8 putative transcriptional units) in wild-type, *ptmF*, and *ptmE* mutant strains of *S. pactum* by RT-PCR. The results

showed that disruption of *ptmF* or *ptmE* abolished transcriptional activation of genes from all 8 operons, which suggest that the PtmF-PtmE system controls the expression of the whole gene clusters. However, since PtmE is not a DNA-binding protein, its mechanism of transcriptional activation remains unknown. In addition, it may be premature to assume that only PtmF and PtmE are responsible for regulating the biosynthesis of pactamycin, as some other genes within the cluster may play a role as well. For example, a number of genes in the cluster, e.g., *orf9*, *orf11*, *orf14*, *orf15*, *orf16*, and *orf19*, are also assigned as putative regulatory genes. Orf11 shares high homology with the extracytoplasmic function (ECF) subfamily of RNA polymerase sigma factors. The location of *orf11* near the structural genes in the cluster suggests that this sigma factor gene may be involved in the regulation of pactamycin gene expression. Orf14 and Orf15 are hypothetical proteins that have low identity to the translation initiation factor (IF-2) from *Frankia alni* ACN4a and *Streptomyces avermitilis* MA-4680, respectively. Their function related to pactamycin biosynthesis is unclear but may be involved in regulating the production of biosynthetic enzymes. On the other hand, Orf9, Orf16, and Orf19 are highly related to the family of ATP-dependent (DEAD-box) RNA helicases. However, inactivation of *orf9*, *orf11*, *orf14*, *orf16*, and *orf19* did not significantly affect pactamycin production (unpublished data).

In asukamycin biosynthesis in *Streptomyces nodosus* subsp. *asukaensis*, at least six putative pathway-specific regulatory proteins, including a member of the SARP family (AsuR5), govern the biosynthesis of asukamycin at various intensities (Xie et al. 2012). Similar to *ptmF*, inactivation of *asuR5* completely abolished the production of asukamycin, and introduction of a second copy of the gene to the producing strain did not improve the production of the antibiotic. However, introduction of the *asuR1-asuR4* operon into *S. nodosus* did improve the production of asukamycin by 14-fold. Unfortunately, this was not the case with the *ptmG-ptmF-ptmE* operon, as the introduction of a second copy of this operon did not increase the production of pactamycin (Fig. 6). Therefore, it is believed that in addition to PtmE and PtmF there are other systems in *S. pactum* that play a role in balancing the homeostasis of the cell and controlling the production of pactamycin.

We found that pactamycin production is affected by inorganic phosphate concentration. Comparative experiments using various concentrations of phosphate added to the cultures of *S. pactum* showed that almost no pactamycin was produced when initial phosphate concentration is higher than 2 mM (Fig. 7). This does not only apply to pactamycin biosynthesis, but also to those of NFAT-133 and piericidins. At high phosphate concentrations the transcription of *ptmF* and *ptmE* were also significantly diminished, which in turn reduced the expression of the biosynthetic genes and the production of pactamycin.

The effects of phosphate on secondary metabolite biosynthesis mediated by a two-component PhoR-PhoP system have been reported in many *Streptomyces* species (Martin and Liras 2010, Martin et al. 2011). A set of genes encoding a two-component PhoR-PhoP system was also found in *S. pactum*. As expected, inactivation of the *phoP* gene in *S. pactum* diminished the effects of high phosphate concentration on pactamycin biosynthesis (Fig. 8b). Interestingly, in both *S. pactum* *ptmH* and *ptmH/phoP::aac(3)IV* mutants, a phosphate concentration of 10 mM does not seem to affect the production of NFAT-133. On the contrary, such amount of phosphate in the medium has effectively inhibited piericidin

biosynthesis even when the *phoP* gene inactivated, underscoring the complexity of regulatory systems in secondary metabolism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Compliance to Ethical Standards

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Fig. 1.

Chemical structures and biosynthetic gene cluster of pactamycin. **(a)** Chemical structures of pactamycin and its congeners. **(b)** the pactamycin biosynthetic gene cluster.

Fig. 2. Generation and analysis of *ptmF* and *ptmE* mutants. (a) Cloning strategy for construction of *ptmF*. (b) PCR analysis of wild-type, *ptmF* and *ptmE* mutant strains of *S. pactum*. (c)-(d) HPLC and MS comparisons between wild-type and the *ptmF* and *ptmE* mutants.

Fig. 3.

HPLC and mass spectrometry comparisons of *ptmF*, *ptmE*, and those complemented by the *ptmG-ptmF-ptmE* operon. (a) Partial HPLC chromatograms of *ptmF* and *ptmE* show complete shutdown of pactamycin biosynthesis in *ptmF* and *ptmE* mutants; however, gene complementation with the *ptmG-ptmF-ptmE* operon led to full restoration of pactamycin biosynthesis in these mutants. (b) Partial mass spectrometry spectra of the extracts from the *ptmF*, *ptmE* mutants and those complemented by the *ptmG-ptmF-ptmE* operon.

Fig. 4.

Growth-phase-dependent biosynthesis of pactamycin. **(a)** Growth curve of *S. pactum* wild-type (red) and *ptmF* mutant (blue). **(b)** Measurement of pactamycin analogues production in *S. pactum* wild-type at different growth stages. **(c)** Time courses of antibiotic production in *S. pactum* wild-type and *ptmF* mutant strains as evaluated by bioassay against *S. aureus*. **(d)** Time courses of transcription of representative pactamycin biosynthetic genes.

Fig. 5.

RT-PCR detection of the transcripts of the pactamycin biosynthetic genes in *S. pactum* wild-type (WT), *ptmE* (ΔE) and *ptmF* (ΔF) mutant strains. 16S rRNA was used as control. M, DNA ladder.

Fig. 6. HPLC comparisons of TM-026 production in *S. pactum* $\Delta ptmH$ and *S. pactum* $\Delta ptmH$ containing a second copy of the *ptmG-ptmF-ptmE* operon. Red arrow, TM-026; black star, an unknown product.

Fig. 7.

Effect of initial phosphate concentration on the biosynthesis of pactamycin. HPLC analysis of pactamycin analogues production with different initial phosphate concentration (**a**).

Transcriptions of pactamycin biosynthetic genes were repressed by increasing phosphate concentration (**b**).

Fig. 8.

Effects of inorganic phosphate concentration on TM-026 production in *S. pactum* *ptmH* and *ptmH/phoP::aac(3)IV*. **(a)** Chromatograms of TM-026 production by *S. pactum* *ptmH* grown in various phosphate concentrations. **(b)** Chromatograms of TM-026 production by *S. pactum* *ptmH/phoP::aac(3)IV* grown in various phosphate concentrations. Red arrow, TM-026; black star, an unknown metabolite.