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Improvement of pristinamycin I (PI) production in *Streptomyces pristinaespiralis* by metabolic engineering approaches

Jiali Meng [a, b, 1], Rongrong Feng [a, 1], Guosong Zheng [a], Mei Ge [c], Yvonne Mast [d], Wolfgang Wohlleben [d, **], Jufang Gao [b], Weihong Jiang [a, e, ***], Yinhua Lu [a, *]

[a] Key Laboratory of Synthetic Biology, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, 200032, China
[b] Shanghai Normal University, Shanghai, 200234, China
[c] Shanghai Laiyi Center for Biopharmaceuticals R&D, Shanghai, 201203, China
[d] Mikrobiologie/Biotechnologie, Interfakultäres Institut für Mikrobiologie und Infektionsmedizin, Fakultät für Biologie, Eberhard Karls Universität Tübingen, Auf der Morgenstelle 28, D-72076, Tübingen, Germany
[e] Jiangsu National Synergetic Innovation Center for Advanced Materials, SICAM, Nanjing, 210009, China

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ABSTRACT

Pristinamycin, produced by *Streptomyces pristinaespiralis*, which is a streptogramin-like antibiotic consisting of two chemically unrelated components: pristinamycin I (PI) and pristinamycin II (PII), shows potent activity against many antibiotic-resistant pathogens. However, so far pristinamycin production titers are still quite low, particularly those of PI. In this study, we constructed a PI single component producing strain by deleting the PII biosynthetic genes (*snaE1* and *snaE2*). Then, two metabolic engineering approaches, including deletion of the repressor gene *papR3* and chromosomal integration of an extra copy of the PI biosynthetic gene cluster (BGC), were employed to improve PI production. The final engineered strain ΔPIIΔ*papR3*/PI produced a maximum PI level of 132 mg/L, with an approximately 2.4-fold higher than that of the parental strain *S. pristinaespiralis* HCCB10218. Considering that the PI biosynthetic genes are clustered in two main regions in the 210 kb "supercluster" containing the PI and PII biosynthetic genes as well as a cryptic polyketide BGC, these two regions were cloned separately and then were successfully assembled into the PI BGC by the transformation-associated recombination (TAR) system. Collectively, the metabolic engineering approaches employed is very efficient for strain improvement in order to enhance PI titer.

1. Introduction

Pristinamycin is a streptogramin-like antibiotic consisting of a mixture of two chemically unrelated components: pristinamycin I (PI), a branched cyclic hexadepsipeptide (Fig. 1A) and pristinamycin II (PII), a polyunsaturated cyclo-peptide macrolactone [1]. PI and PII are produced by *Streptomyces pristinaespiralis* in a ratio of 30:70 and are synthesized by non-ribosomal peptide synthetases (NRPSs) and hybrid polyketide synthase (PKS)/NRPS enzymes, respectively [2]. The combination of the two compounds shows a strong synergistic effect, with a bactericidal activity 100-fold higher than that of the single components [3]. Pristinamycin is highly active against many antibiotic-resistant pathogens, particularly Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA) and *Enterococcus faecium* (VREF) [4]. Currently, pristinamycin and its derivatives are used as therapeutics to treat severe bacterial infections caused by multi-drug resistant pathogens. However, so far the production titers of pristinamycin (both PI and PII) by *S. pristinaespiralis* are still quite low and require to be improved for scale-up industrial production.

Because of the toxic effects of the synergistic active PI/PII combination on mycelial growth of *S. pristinaespiralis* [5], as well as

* Corresponding author.
** Corresponding author.
*** Corresponding author. Key Laboratory of Synthetic Biology, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, 200032, China.
E-mail addresses: wolfgang.wohlleben@biotech.uni-tuebingen.de (W. Wohlleben), whjiang@sibs.ac.cn (W. Jiang), yhlu@sibs.ac.cn (Y. Lu).
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[1] Jiali Meng and Rongrong Feng contributed equally to this work.

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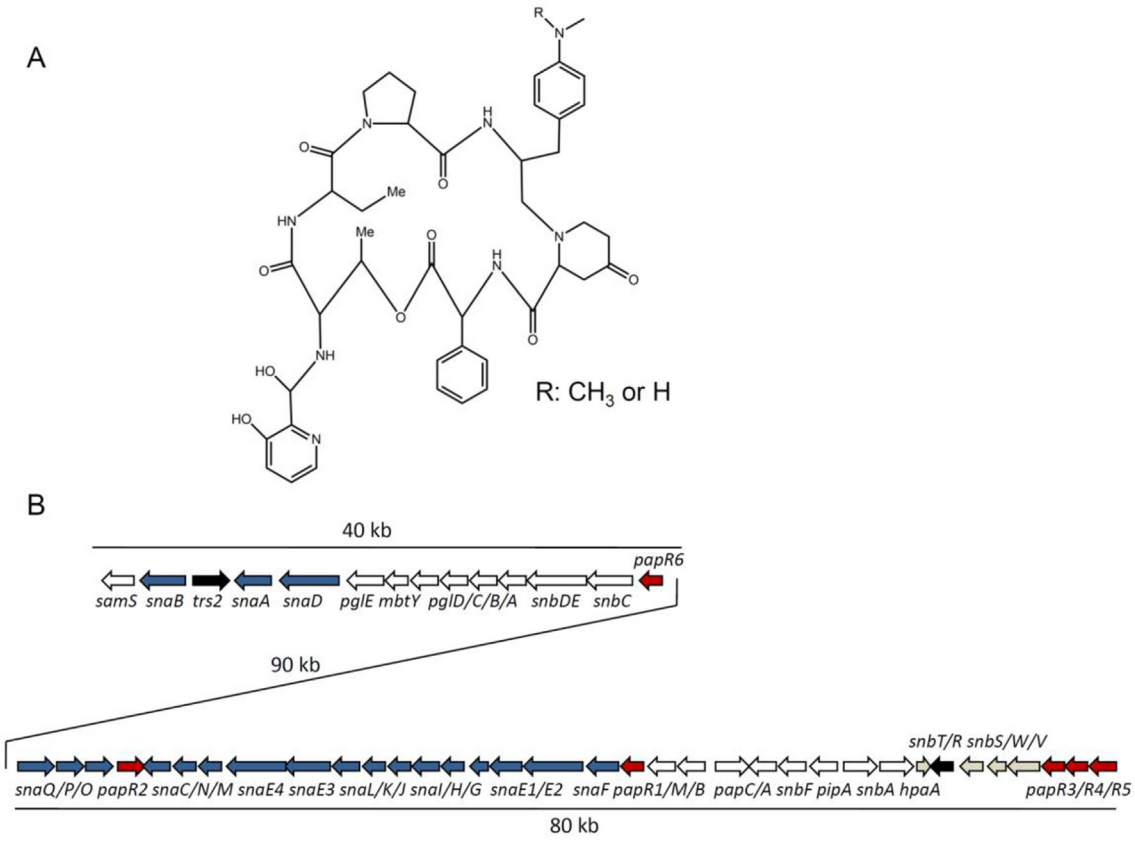

Fig. 1. The chemical structure of pristinamycin I (PI) and organization of the pristinamycin biosynthetic gene cluster (BGC) in *S. pristinaespiralis*. A. The PI chemical structure. **B**. PI and PII biosynthetic genes are indicated as white and blue arrows, respectively. Regulatory genes are shown as red arrows. Genes for resistance and of general function are shown as black arrows. Genes of unknown function are indicated as grey arrows. PI and PII biosynthetic genes are interspersed by approximately 90 kb as indicated.

mutual interference when they are purified, we aimed to generate two high single component (PI or PII) producing strains. Recently, in our research group, markedly increased PII production has been achieved by two metabolic engineering approaches [5,6], including modification of the pathway-specific regulation pathway and multi-copy chromosomal integration of the PII biosynthetic gene cluster (BGC). For these approaches *S. pristinaespiralis* HCCB10218 has been used as the starting strain, which is generated from traditional physical and chemical mutagenesis of the wild-type strain ATCC25486 [7]. By adding a macroreticular resin, the final engineered strain SBJ1005 produced maximum PII titers of approximately 2.2 g/L and 2 g/L in Erlenmeyer flasks and a 5-L bioreactor, respectively, which are the highest PII titers ever reported [6]. However, so far, the construction of high PI-producing strains was not reported yet.

In the past few years, the genes responsible for the biosynthesis of pristinamycin I has been well characterized [2]. They are clustered together with the PII biosynthetic genes as well as a cryptic type II polyketide BGC in a 210 kb "supercluster" [2] (Fig. 1B). In addition, much effort has been made in our understanding of the regulation of pristinamycin biosynthesis. PapR1, R2 and R4, belong to SARP (*Streptomyces* antibiotic regulatory proteins) family regulators and act as activators for both PI and PII biosynthesis, while PapR3 and R5, belonging to TetR family proteins, act as repressors [2,8]. PapR6 is a response regulator-like protein and acts as a pathway-specific regulator of PII biosynthesis [7]. SpbR, a γ-butyrolactone receptor, acts as a global player in the regulation of pristinamycin production but affects also bacterial growth and development [9]. Knowledge on the pristinamycin BGC as well as on the regulation of pristinamycin biosynthesis provides clues for strain improvement by metabolic engineering approaches. Furthermore, in *Streptomyces* various metabolic engineering approaches have been developed [10,11], such as manipulating regulatory pathways, increasing the supply of specific building blocks and amplification of secondary metabolite BGCs of interest, which have been proven as efficient methods for *Streptomyces* strain improvement.

In this study, two PII biosynthetic genes *snaE1* and *E2* (encoding the deduced hybrid PKS/NRPS complex SnaE1 and the predicted PKS SnaE2, respectively) were deleted to yield a sole PI-producing strain. Then, two metabolic engineering approaches, including deletion of the repressor gene *papR3* and adding an extra copy of the PI BGC, were employed to enhance PI production. The final engineered strain produced over two-fold higher PI titers than that of the parental strain *S. pristinaespiralis* HCCB10218, indicating that the strategy employed here is very efficient for PI overproduction.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

All strains and plasmids used in this study are listed in Table 1. *S. pristinaespiralis* HCCB10218 (CGMCC 5486) is a pristinamycin producing strain isolated after traditional mutagenesis (physical and chemical mutations) of the wild-type strain ATCC 25486 [7]. *S. pristinaespiralis* strains were cultivated on RP agar at 30 °C for the

Table 1
Strains and plasmids used in this study.

Strains	Genotype	Source
***E. coli* strains**		
DH5α	F−Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) *U169 deoR recA1 endA1 hsdR17(rk-mk+) phoA supE44 λ- thi-1 gyrA96 relA1*	Gibco-BRL
EPI300	*recA1 endA1 araD139* Δ(*ara, leu*)7697 λ- *rpsL*(Str^r) *trfA dhfr*	Epicentre
ET12567/ pUZ8002	The methylation defective strain ET12567 containing the RP4 derivative plasmid pUZ8002	[18]
S. cerevisiae		
VL6-48	Host strain; *MAT alpha, his3-D200, trp1-D1, ura3-52, lys2, ade2-101, met14, psi + cir0*	ATCC MYA-3666
S. pristinaespiralis		
HCCB10218	Parental strain	[7]
ΔPII	Mutant strain, HCCB10218 with deletion of the PII biosynthetic genes (*snaE1* and *snaE2*)	This study
ΔPIIΔ*papR3*	Mutant strain, HCCB10218 with deletion of both the PII biosynthetic genes (*snaE1* and *snaE2*) and the repressor gene *papR3*	This study
ΔPIIΔ*papR3*/PI	Mutant strain, ΔPIIΔ*papR3* with chromosomal integration of one copy of the assembled PI BGC via site-specific recombination	This study
Plasmids		
pKCcas9dO	CRISPR/Cas9 editing plasmid for deletion of the pathway-specific activator gene *actII-ORF4* of ACT biosynthesis in *S. coelicolor*, *acc(3)IV*, pSG5 *ori*, *tipA*p-Sco*cas9*, j23119p-sgRNA	[14]
pKCcas9dPII	CRISPR/Cas9 editing plasmid for deletion of the PII biosynthetic genes (*snaE1* and *snaE2*), *acc(3)IV*, pSG5 *ori*, *tipA*p-Sco*cas9*, j23119p-sgRNA	This study
pKCcas-*papR3*	CRISPR/Cas9 editing plasmid for *papR3* deletion, *acc(3)IV*, pSG5 *ori*, *tipA*p-Sco*cas9*, j23119-sgRNA	This study
pCAP01	Gene cluster capture vector; ARSH4/CEN6, pUC *ori*, *aph(3)II*, ΦC31 *int/attP*, *oriT* (RP4)	[13]
pCAP-*spr1*	Recombinant plasmid containing the PI BGC region between *pglE* and *snbC* (28 kb) cloned in pCAP01	This study
pCAP-*spr2*	Recombinant plasmid containing the PI BGC region between *papM* and *hpaA* (11 kb) cloned in pCAP01	This study
pCAP-PI	Recombinant plasmid containing the assembled PI BGC cloned in pCAP01	This study

preparation of spore suspensions as well as for intergeneric conjugation [5]. For the analysis of PI production, *S. pristinaespiralis* strains were grown in seed medium and fermentation medium as described previously [2]. *E. coli* strains were grown on Luria-Bertani (LB) agar or in LB broth at 37 °C. DH5α and EPI300 were used as the hosts for plasmid construction and for the propagation of the recombinant plasmid (pCAP-PI) containing the PI BGC, respectively. The methylation defective *E. coli* ET12567/pUZ8002 (a RK2 derivative) was used as the donor in the experiments of intergeneric conjugation between *E. coli* and *S. pristinaespiralis* [12]. *Saccharomyces cerevisiae* VL6-48 was grown in YPD medium [13] and was used for cloning and assembly of the PI BGC. When necessary, antibiotics, such as kanamycin (50 μg/ml), apramycin (50 μg/ml), chloramphenicol (33 μg/ml) or/and ampicillin (100 μg/ml) were added.

2.2. Construction of *S. pristinaespiralis* mutants

The mutants ΔPII and ΔPIIΔ*papR3*, with the respective deletion of the PII biosynthetic genes (*snaE1/E2*, totally 18,781 bp), as well as both, *snaE1/E2* and *papR3* repressor gene deletion (1575 bp) were constructed on the basis of the parental strain HCCB10218 using the CRISPR/Cas9-mediated genome editing method as described previously [14]. Here, we present ΔPII as an example to give a brief introduction of the procedure for mutant construction: The sgRNA transcription cassette was obtained by PCR with the primer pair snaEgRNA-fw/gRNA-rev and the plasmid pKCcas9dO as the template. Two homologous arms (1921 and 1973 bp) were amplified from the genomic DNA of *S. pristinaespiralis* HCCB10218 using the primer pairs snaE-up-fw/rev and snaE-down-fw/rev, respectively. The three DNA fragments were ligated together by an overlapping PCR using the primers snaEsgRNA-fw/snaE-down-rev. The obtained PCR product was cloned as *Spe*I/*Hin*dIII−restricted fragment into the plasmid pKCcas9dO, which was pretreated with the same two restriction enzymes, resulting in pKCcas-PII. pKCcas-PII was introduced into HCCB10218 by intergeneric conjugation between ET12567/pUZ8002 and *S. pristinaespiralis*. The mutants with correct deletion of *snaE1/E2* were identified by colony PCR with the primer pairs, JsnaE-inner-fw/rev and JsnaE-outer-fw/rev, respectively, followed by DNA sequencing. The obtained mutant was named as ΔPII. Using the ΔPII mutant as the starting strain, the ΔPIIΔ*papR3* mutant was constructed similarly by deleting the repressor gene *papR3*. The primers used are listed in Table 2.

2.3. Cloning of the PI BGC

The PI biosynthetic genes are located in two main regions, including region 1 (named as *spr1*, from *pglE* to *snbC*, approximately 28 kb) and region 2 (named as *spr2*, from *papM* to *hpaA*, approximately 11 kb), as presented in Fig. 1. *spr1* and *spr2* were cloned separately (2.3.1) and then assembled together (2.3.2). It should be noted that the *samS* gene possibly involved in PI biosynthesis, which is located upstream of the PII biosynthetic gene *snaB*, was not included. The primers used are listed in Table 2.

2.3.1. Cloning of two partial PI BGC (spr1 and spr2) separately

The *spr1* region was cloned by the CRISPR/Cas9-mediated TAR technology [15]. Two sgRNAs directed to the upstream region of *snbC* and downstream region of *pglE* were designed. The *spr1* sgRNAs transcription cassettes with T7 promoter sequence were amplified from the plasmid pKCcas9dO with the primer pairs, spr1gRNA1-fw/gRNA-rev and spr1gRNA2-fw/gRNA-rev, respectively. Then, *in vitro* transcription was performed using the MEGAScriptTM Kit (Ambion), followed by purification with the MEGAClear™ Kit (Ambion). Finally, CRISPR/Cas9 (Tolo Biotech., Shanghai, China)-mediated genomic DNA digestion was conducted as described previously [15]. The enzymatically fragmented genomic DNA was purified by ethanol precipitation.

The *spr1* capture vector was constructed as follows. Two homologous arms (approximately 1 kb) corresponding to flanking regions of *spr1* were amplified from *S. pristinaespiralis* HCCB10218 genomic DNA with two primer pairs, spr1up-fw/rev and spr1down-fw/rev, respectively, and introduced into the *Spe*I/*Kpn*I-digested plasmid pCAP01, generating the *spr1* capture plasmid pCAP01-*spr1*. Then, spheroplast cells of *S. cerevisiae* VL6-48 were transformed with 1 μg linearized pCAP01-*spr1* (treated by *Eco*RI) and 2−3 μg Cas9-treated HCCB10218 genomic DNA. Desired transformants was screened on SD-Trp agar. The positive clones were verified by PCR. Two primer pairs, including Jspr1-A-fw/rev (amplifying the joint region between the vector and *spr1*) and Jspr1-B-fw/rev (amplifying the inner regions of *spr1*) were used. The putative positive plasmids were extracted from yeast and then transformed into

Table 2
Primers used in this study.

Primers	Sequences (5′-3′)
Amplification of two sgRNAs used for deletion of the PII biosynthetic genes (*snaE1* and *snaE2*) and *papR3*, respectively (N20, guide sequences are underlined and the restriction enzyme sites are in italics)	
snaE-gRNA-fw	AGCTAGCTCAGTCCTAGGTATAAT*ACTAGT*TGGGTCCGTAGACGTTCCACGTTTTAGAGCTAGAAATA
papR3-gRNA-fw	AGCTAGCTCAGTCCTAGGTATAAT*ACTAGT*CGGCGCGTACGACGGGGTCCGTTTTAGAGCTAGAAATACTCAAAAAAAGCACCGACTCGG
gRNA-rev	CTCAAAAAAAGCACCGACTCGG
Amplification of upstream and downstream homologous arms (the sequences for overlapping PCR are underlined and the enzyme sites are in italics)	
snaE-up-fw	CACCGAGTCGGTGCTTTTTTTGAGGCCGTAGGCGTTGTTGAGG
snaE-up-rev	CCGCACCAACGGCTACACC
snaE-down-fw	GGCACGGTGTAGCCGTTGGTGCGGGAGGAAGGCGGGGAAGGTC
snaE-down-rev	CGTTGTAAAACGACGGCCAGTGCC*AAGCTT*GCACCCTGGACACCTGGCT
papR3-up-fw	AAGTGGCACCGAGTCGGTGCTTTTTTTGAGCGAAACCCGACGCACGAT
papR3-up-rev	CGTTCCCGTCGGCGAGTCATC
papR3-down-fw	CATGCGGGATGACTCGCCGACGGGAACGGCGTGGGTGGTTGCCTCTG
papR3-down-rev	CGTTGTAAAACGACGGCCAGTGCC*AAGCTT*CGAGCGGATGCTGCACGAC
Verification of the deletion of the PII biosynthetic genes (*snaE1* and *snaE2*) and *papR3*	
JsnaE-inner-fw	GTGTCAGGGGCGGGGAGGAA
JsnaE-inner-rev	CTGCACGGCGTCGTCCACG
JsnaE-outer-fw	CGGGCGGACAGGAACACCA
JsnaE-outer-rev	ACATCCGCACCGCCTTCG
JpapR3-inner-fw	ACCATCGGTGTACGGCTTCT
JpapR3-inner-fw	GGACGCCACCCATGTGCTGA
JpapR3-outer-fw	GTGTCGTCGTGGGCAGGTGT
JpapR3-outer-fw	CGGCTATCTGCTGAACACCTCC
Amplification of sgRNAs for Cas9-mediated *in vitro* DNA digestion (N20, guide sequences are underlined and T7 promoter sequences are in italics)	
spr1-gRNA1-fw	*GACTGACACTGATAATACGACTCACTATA*GGACGAAGGTGCAGTTGAAGTTTTAGAGCTAGAAATAGC
spr1-gRNA2-fw	*GACTGACACTGATAATACGACTCACTATA*GGTGCGCAAGGAACTGGAGTTTTAGAGCTAGAAATAGC
gRNA-rev	CTCAAAAAAAGCACCGACTCGG
Amplification of upstream and downstream homologous arms for the construction of the *spr1* and *spr2* capture vectors (the overlapping sequences designed for the assembly of *spr1* and *spr2* are underlined and the enzyme sites are in italics)	
spr1UP-fw	G*ACTAGT*TACGTGCCGAGGGCCTTGAGGTA
spr1UP-rev	G*GAATTC*CATATGGAGTCCGAGGACACCGCGCGAAGC
spr1DOWN-fw	G*GAATTC*CATATGTCAGCGCAGGCCGGTCTCGAAGGCGTA
spr1DOWN-rev	G*GGGTACC*GCTCTAGACCGCCCTGTGCGAACTGCTGGAACCCGACGACCTGCTCTTAGTTCCTCGCGCAGCCACAGAT
spr2UP-fw	G*ACTAGT*ACCAGCTGATCGCGGTCCATCTGTGGCTGCGCGAGGAACTAAGAGCAGGTCGTCGGGTTC
spr2UP-rev	G*GAATTC*CATATGATCCTCCAGCTGCGCGAACTGA
spr2DOWN-fw	G*GAATTC*CATATGGTATGCCGTTCATCGAAGTG
spr2DOWN-rev	CCG*CTCGAG*ACTACGACCGTCCCGTGCCGCGAAG
Verification of direct cloning of spr1 and spr2 as well as of the assembly of PI BGC	
Jspr1-A-fw	CCAGGAAACGGACGAAGCG
Jspr1-A-rev	CCTGACGGGCTTGTCTGCTC
Jspr1-B -fw	GAGGGAGTCGTAGGTCTGCTGC
Jspr1-B- rev	GAGCCCTACCAGCACATCGTC
Jspr2-A-fw	GGACCCGTTGGCAGGAAGCA
Jspr2-A-rev	ACACGGCTCCTACCAACTCG
Jspr2-B-fw	TGGTCCTTCAGGCACAGCA
Jspr2-B-rev	CGACCACATCACCCTCAAGACC

E. coli EPI 300 for propagation. Direct cloning of the *spr1* region was verified by restriction enzyme analysis, resulting in the plasmid pCAP-*spr1*.

Direct cloning of the *spr2* region was carried out using traditional TAR method [13], similarly as that of the CRISPR/Cas9-mediated TAR used for *spr1* cloning. The only difference is that genomic DNA of HCCB10218 was digested with *Xba*I and *Nde*I but not Cas9. The obtained plasmid was named pCAP-*spr2*.

2.3.2. Assembly of the PI BGC

Assembly of the PI BGC was performed by the TAR method [13]. To assemble *spr1* and *spr2* into the PI BGC, we designed a 80-bp overlapping region between *spr1* and *spr2*. The capture vector for *spr1* and *spr2* assembly was constructed as that of pCAP-*spr1*. The primer pairs used for the amplification of the two homologous arms were spr1up-fw/rev and spr2down-fw/rev. pCAP-*spr1* and pCAP-*spr2* were digested with *Spe*I/*Kpn*I and *Spe*I/*Xho*I, respectively, to isolate *spr1* and *spr2* large DNA fragments. Correct assembly between *spr1* and *spr2* was confirmed by colony PCR (using primer pairs of Jspr1-B-fw/rev and Jspr2-B-fw/rev) and restriction enzyme analysis, resulting in the plasmid pCAP-PI, which contained the expected PI BGC.

2.4. S. pristinaespiralis fermentation and quantitative analysis of PI production

S. pristinaespiralis fermentation was conducted according to the method described by Mast et al. [2]. Briefly, *S. pristinaespiralis* strains were cultured in 25 ml of seed medium in 250 ml flasks at 27 °C on a rotary shaker (240 rpm). After 40–44 h, cultures (2 ml each) were harvested to inoculate 3 × 25 ml of fermentation medium in 250 ml-volume flasks that were incubated at 27 °C (240 rpm). Fermentation cultures (0.5 ml each) were collected at five different time points (30, 48, 60, 72 and 96 h), followed by extraction with the same volume of acetone for 60 min. The mixtures were centrifuged at 4000 rpm for 10 min. The supernatants were analyzed for PI production by high-performance liquid chromatography (HPLC) according to the method described previously [16]. The elution time for PI_A (the main component of PI) was approximately 9.9 min. Purified PI_A (98%,

provided by Shanghai Liyi Co. Ltd, Shanghai, China) was used to make standard curves.

3. Results

3.1. Construction of a sole pristinamycin I (PI)-producing strain

To construct a sole PI-producing strain, we generated the mutant ΔPII on the basis of the parental strain *S. pristinaespiralis* HCCB10218 with an in-frame deletion of two PII biosynthetic genes, *snaE1* and *snaE2*, which encode the deduced hybrid PKS/NRPS complex SnaE1 and the predicted PKS SnaE2 [2]. The correct deletion of these two genes was verified by PCR (Fig. 2A) and DNA sequencing (data not shown). Deletion of *snaE1/E2* has no effect on bacterial growth (data not shown). Fermentation cultures of HCCB10218 and the ΔPII mutant were collected at five different time points for the analysis of pristinamycin (both PI and PII) production titers by HPLC. These analyses showed that compared with HCCB10218 that could produce both, PI and PII, the ΔPII mutant only formed PI (Fig. 2B). Furthermore, to our surprise, we found that deletion of *snaE1/E2* led to approximately 20–40% reduced PI production titers in comparison to the parental strain HCCB10218 (Fig. 2C).

3.2. Deletion of the repressor gene papR3 results in enhanced PI production

In *S. pristinaespiralis*, the TetR-family regulator PapR3 has been identified as repressor of PI and PII biosynthesis [2,5,8]. Deletion of *papR3* has been shown to result in a markedly enhanced PI and PII production [5]. To further increase PI production in ΔPII, we constructed the mutant ΔPIIΔ*papR3* with an in-frame deletion of *papR3* on the basis of the ΔPII mutant. The correct deletion of *papR3* was confirmed by colony PCR (Fig. 3A) and DNA sequencing (data not shown). Bacterial growth was not affected upon deletion of *papR3* (data not shown). Fermentation cultures of HCCB10218 and the mutants ΔPII and ΔPIIΔ*papR3* were collected at five different time points for PI production analysis. The results showed that deletion of *papR3* on the basis of ΔPII mutant resulted in a markedly enhanced PI production. The ΔPIIΔ*papR3* mutant produced a maximum PI level of 90 mg/L, showing a 1.4-fold higher than the parental strain HCCB10218 (approximately 38 mg/L) (Fig. 3B).

3.3. Adding an extra copy of the PI BGC leads to further increase of PI production

Another efficient strategy to optimize antibiotic production yield is to increase the copy number of secondary metabolite BGCs

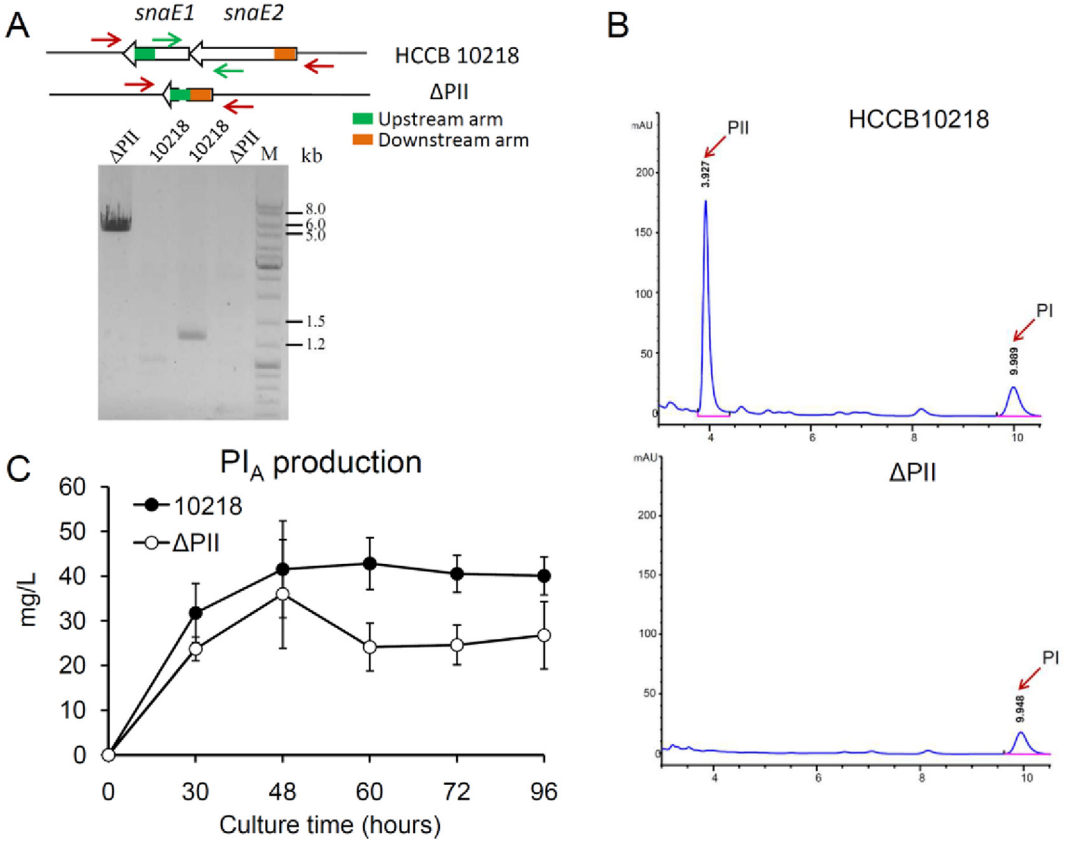

Fig. 2. Verification of the sole PI component producing strain ΔPII. **A**. Verification of the deletion of the PII biosynthetic genes (*snaE1* and *snaE2*) by colony PCR. The primer pair located upstream of *snaE1* and downstream of *snaE2* (JsnaE-outer-fw/rev) is indicated as red arrows. The expected band size for the ΔPII mutant is 4152 bp. In the parental strain HCCB10218, due to too large DNA fragment (>20 kb), no band could be amplified by PCR. The primer pair located within the *snaE1* and *snaE2* ORF (JsnaE-inner-fw/rev) is indicated as green arrows. The expected size for the parental strain HCCB10218 is 1220 bp. For the ΔPII mutant sample, where *snaE1* and *snaE2* are deleted, no band was amplified by PCR. **B**. Pristinamycin production of the parental strain HCCB10218 and the ΔPII mutant analyzed by HPLC. **C**. Effect of the *snaE1/E2* deletion on PI production. Fermentation cultures of the ΔPII mutant and the parental strain HCCB10218 were collected at five time points as indicated. Fermentation analysis was performed in triplicate and was repeated twice. Error bars indicate the standard deviations for three biological replicates.

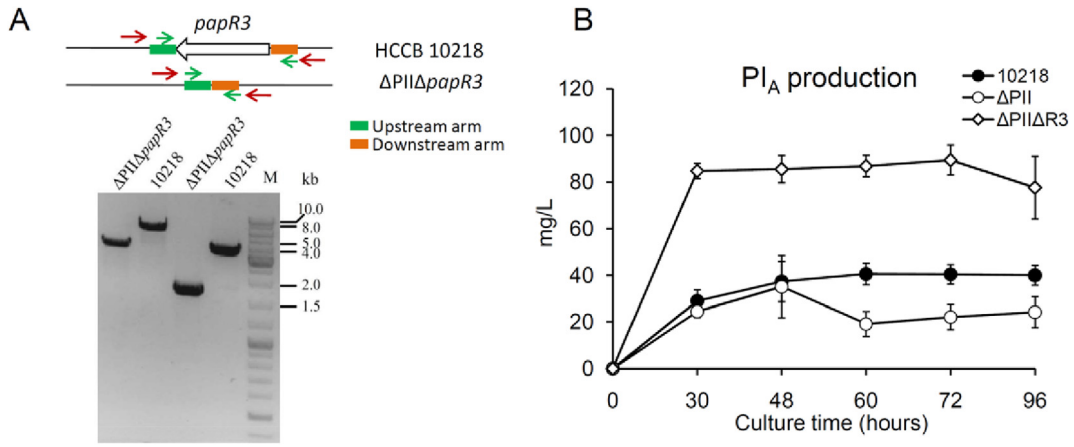

Fig. 3. Verification of the ΔPIIΔ*papR3* mutant with deletion of two PII biosynthetic genes *snaE1/E2* and the repressor gene *papR3*. A. Verification of *papR3* deletion by colony PCR. The primer pairs located outside and inside the two homologous arms (JpapR3-outer-fw/rev and JpapR3-inner-fw/rev) are indicated as red and green arrows, respectively. The expected band sizes amplified by red primers (outside) for the ΔPII mutant and HCCB10218 are 3287 and 4862 bp, respectively. The primer pair amplified by green primers (inside) are 1685 and 3260 bp for the ΔPII mutant and HCCB10218, respectively. **B**. Effect of *papR3* deletion on PI production. Fermentation cultures of three strains, including HCCB10218, ΔPII and ΔPIIΔ*papR3*, were collected at five time points as indicated. Fermentation analysis was performed in triplicate and was repeated twice. Error bars indicate the standard deviations for three biological replicates.

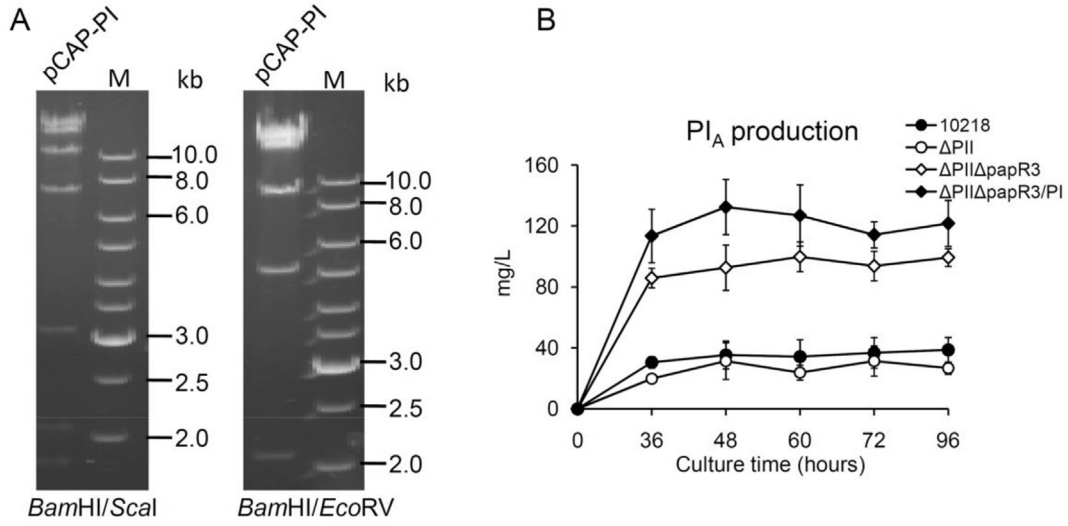

Fig. 4. Effect on PI production after chromosomal integration of an extra copy of the PI BGC into ΔPIIΔ*papR3*. A. Verification of the plasmid pCAP-PI containing the assembled PI BGC by restriction enzyme analysis. Two groups of enzymes were used as indicated. The expected sizes after *Bam*HI/*Sca*I digestion are 1780, 2008, 3013, 6917, 9666, 12771 and 16339 bp. The expected sized after *Bam*HI/*Eco*RV digestion are 2008, 4793, 8447, 14809 and 22437 bp. **B**. Effects on PI production after chromosomal integration of an extra copy of the PI BGC into ΔPIIΔ*papR3*. Fermentation cultures of the four strains, including HCCB10218, ΔPII, ΔPIIΔ*papR3* and ΔPIIΔ*papR3*/PI, were collected at five time points as indicated. Fermentation analysis was performed in triplicate and was repeated twice. Error bars indicate the standard deviations for three biological replicates.

[5,6,10]. Thus, we applied this metabolic engineering strategy in order to increase PI production in ΔPIIΔ*papR3*. To achieve this, the first step is to clone the PI BGC. The PI biosynthetic genes are scattered in two main regions, namely, region 1 (named as *spr1*, from *snbC* to *pglE*, approximately 28 kb) and region 2 (named as *spr2*, from *papM* to *hpaA*, approximately 11 kb) (Fig. 1). These two regions were successfully cloned using the TAR method [13,15] and then assembled into the PI BGC to yield the plasmid pCAP-PI. The correctness of the pCAP-PI was confirmed by PCR (data not shown) and restriction enzyme analysis using *Bam*HI/*Sca*I and *Bam*HI/*Eco*RV, respectively (Fig. 4A).

3.3.1. *Introduction of pCAP-PI into the ΔPIIΔpapR3 mutant further increases PI production yield*

We introduced the plasmid pCAP-PI (containing the PI BGC) into the mutant ΔPIIΔ*papR3* by the ΦC31 integrase-mediated site-specific DNA recombination. This resulted in strain ΔPIIΔ*papR3*/PI, where an extra copy of the PI BGC is present. The engineered strain showed similar morphological development and bacterial growth as HCCB10218 and ΔPIIΔ*papR3* (data not shown). Fermentation cultures of HCCB10218, ΔPII, ΔPIIΔ*papR3* andΔPIIΔ*papR3* were taken at five different time points for PI production analysis. The HPLC analyses demonstrated that the introduction of an extra copy of the PI BGC led to a further increase of PI production. The ΔPIIΔ*papR3*/PI mutant produced a maximum PI level of 132 mg/L, showing approximately 30% and 240% higher than those of the ΔPIIΔ*papR3* mutant and HCCB10218, respectively (Fig. 4B). Therefore, it could be concluded that the combination of the two metabolic engineering approaches employed is very efficient for the improvement of PI production by *S. pristinaespiralis*.

4. Discussion

In this study, a high pristinamycin I (PI) single component-producing strain was constructed by deleting the PII biosynthetic genes combined with two metabolic engineering approaches, including the deletion of the repressor gene *papR3* and the addition of an extra copy of the PI BGC. The final engineered strain ΔPIIΔ*papR3*/PI produced a maximum PI level of 132 mg/L, showing an approximately 2.4-fold higher than that of the parental strain HCCB10218. However, the PI titer of ΔPIIΔ*papR3*/PI is still insufficient for industrial production. Therefore, other metabolic engineering strategies, which have been widely proven as efficient methods for *Streptomyces* strain improvement [10], such as increasing precursor supply, overexpression of activator genes and deletion of competing pathways (other secondary metabolite BGCs) could be included to further enhance PI titer.

Interestingly, we found that deletion of the PII biosynthetic genes resulted in 20–40% reduced PI production. In *S. pristinaespiralis*, pristinamycin biosynthesis is under complex regulation involving up to seven cluster-situated regulators, including SpbR and additional six regulators (PapR1-PapR6) [8], as well as regulators located outside of the pristinamycin BGC, such as AtrA-p or Spy1 [16,17]. We propose that PII may act as a coactivator or an inducer for PI biosynthesis. In the presence of PII, activators could bind to the promoter region of target genes and activate PI production, whereas repressors would dissociate from target promoter and result in the derepression of PI gene expression. Accordingly, loss of PII production would lead to a downregulation of PI biosynthetic gene expression and thus decrease PI production. So far, the detailed mechanisms of pristinamycin biosynthesis control remain to be elucidated. However, a better understanding of the regulatory principles of pristinamycin biosynthesis would be greatly beneficial to perform further strain improvement for PI overproduction in *S. pristinaespiralis*.

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References

[1] Barriere JC, Berthaud N, Beyer D, Dutka-Malen S, Paris JM, Desnottes JF. Recent developments in streptogramin research. Curr Pharm Des 1998;4:155–80.
[2] Mast Y, Weber T, Golz M, Ort-Winklbauer R, Gondran A, Wohlleben W, et al. Characterization of the 'pristinamycin supercluster' of *Streptomyces pristinaespiralis*. Microb Biotechnol 2011;4:192–206.
[3] Di Giambattista M, Chinali G, Cocito C. The molecular basis of the inhibitory activities of type A and type B synergimycins and related antibiotics on ribosomes. J Antimicrob Chemother 1989;24:485–507.
[4] Mast Y, Wohlleben W. Streptogramins - two are better than one! Int J Med Microbiol 2014;304:44–50.
[5] Li L, Zhao Y, Ruan L, Yang S, Ge M, Jiang W, et al. A stepwise increase in pristinamycin II biosynthesis by *Streptomyces pristinaespiralis* through combinatorial metabolic engineering. Metab Eng 2015;29:12–25.
[6] Li L, Zheng G, Chen J, Ge M, Jiang W, Lu Y. Multiplexed site-specific genome engineering for overproducing bioactive secondary metabolites in actinomycetes. Metab Eng 2017;40:80–92.
[7] Dun J, Zhao Y, Zheng G, Zhu H, Ruan L, Wang W, et al. PapR6, a putative atypical response regulator, functions as a pathway-specific activator of pristinamycin II biosynthesis in *Streptomyces pristinaespiralis*. J Bacteriol 2015;197:441–50.
[8] Mast Y, Guezguez J, Handel F, Schinko E. A complex signaling cascade governs pristinamycin biosynthesis in *Streptomyces pristinaespiralis*. Appl Environ Microbiol 2015;81:6621–36.
[9] Folcher M, Gaillard H, Nguyen LT, Nguyen KT, Lacroix P, Bamas-Jacques N, et al. Pleiotropic functions of a *Streptomyces pristinaespiralis* autoregulator receptor in development, antibiotic biosynthesis, and expression of a superoxide dismutase. J Biol Chem 2001;276:44297–306.
[10] Zhang MM, Wang Y, Ang EL, Zhao H. Engineering microbial hosts for production of bacterial natural products. Nat Prod Rep 2016;33:963–87.
[11] Olano C, Lombo F, Mendez C, Salas JA. Improving production of bioactive secondary metabolites in actinomycetes by metabolic engineering. Metab Eng 2008;10:281–92.
[12] Kieser TBM, Butter M, Chater K. Practical *Streptomyces* genetics. Norwich, England: John Innes Foundation; 2000.
[13] Yamanaka K, Reynolds KA, Kersten RD, Ryan KS, Gonzalez DJ, Nizet V, et al. Direct cloning and refactoring of a silent lipopeptide biosynthetic gene cluster yields the antibiotic taromycin A. Proc Natl Acad Sci USA 2014;111:1957–62.
[14] Huang H, Zheng G, Jiang W, Hu H, Lu Y. One-step high-efficiency CRISPR/Cas9-mediated genome editing in *Streptomyces*. Acta Biochim Biophys Sin (Shanghai) 2015;47:231–43.
[15] Lee NC, Larionov V, Kouprina N. Highly efficient CRISPR/Cas9-mediated TAR cloning of genes and chromosomal loci from complex genomes in yeast. Nucleic Acids Res 2015;43:e55.
[16] Wang W, Tian J, Li L, Ge M, Zhu H, Zheng G, et al. Identification of two novel regulatory genes involved in pristinamycin biosynthesis and elucidation of the mechanism for AtrA-p-mediated regulation in *Streptomyces pristinaespiralis*. Appl Microbiol Biotechnol 2015;99:7151–64.
[17] Jin Q, Yin H, Hong X, Jin Z. Isolation and functional analysis of *spy1* responsible for pristinamycin yield in *Streptomyces pristinaespiralis*. J Microbiol Biotechnol 2012;22:793–9.
[18] Gust B, Challis GL, Fowler K, Kieser T, Chater KF. PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc Natl Acad Sci USA 2003;100:1541–6.