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A stepwise increase in pristinamycin II biosynthesis by *Streptomyces pristinaespiralis* through combinatorial metabolic engineering

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ABSTRACT

Pristinamycin, which is a streptogramin antibiotic produced by *Streptomyces pristinaespiralis*, contains two chemically unrelated compounds, pristinamycin I (PI) and pristinamycin II (PII). Semi-synthetic derivatives of PI and PII have been approved for use in human medicine to treat a broad range of drug-resistant pathogens. In this study, we design and implement a combinatorial metabolic engineering strategy for improving PII production. First, an extra copy of the PII biosynthetic gene cluster, which was assembled using a modified Gibson assembly method for cloning large DNA fragments with high GC contents, was introduced into a high-producing strain *S. pristinaespiralis* HCCB10218. This duplication of the PII biosynthetic gene cluster resulted in a maximum increase in PII titer by 45%. Second, all seven cluster-situated regulatory genes (from *papR1* to *papR6* and *spbR*) were systematically manipulated. Higher PII titers were achieved by deleting either one of the two repressor genes *papR3* or *papR5* in combination with overexpression of both activator genes *papR4* and *papR6*, and the resulting strains Δ*papR3*+*R4R6* and Δ*papR5*+*R4R6* showed maximum increases in PII production by 99% and 75%, respectively. A combination of the above two different approaches was employed. Integration of the assembled PII gene cluster (BAC-F1F15) into Δ*papR5*+*R4R6* led to the highest PII titer improvement, which was approximately 1.5-fold higher than the parental strain. By adding the macroreticular resin, which can separate pristinamycin *in situ* and thereby lessen end-product feedback inhibition and toxic effects, PII titers of the final engineered strain Δ*papR5*+*R4R6*/BAC-F1F15 reached 1.13 and 1.16 g/L in the Erlenmeyer flask and 5-L bioreactor, respectively, with 5.13- and 5.26-fold improvements over the parental strain. Taken together, this combinatorial strategy is an efficient method to optimize PII biosynthesis of *S. pristinaespiralis* and may be extended to other industrially used streptomycetes for strain improvement.

1. Introduction

Streptomyces bacteria have evolved to produce a vast array of important bioactive secondary metabolites, such as antibiotics, anti-tumor agents and insecticides (Berdy, 2005; Bode and Müller, 2005). Pristinamycin, which is produced by *Streptomyces pristinaespiralis*, is a streptogramin antibiotic that contains two chemically unrelated molecules, pristinamycin I (PI) and pristinamycin II (PII). PI_A and PII_A are the major forms of PI and PII, respectively (Johnston et al., 2002; Mast et al., 2011; Mast and Wohlleben, 2014). The combination of PI and PII shows potent synergistic antibacterial activity that is approximately 100-fold higher compared with treatment with a single component alone (Giambattisti et al., 1989). Quinupristin and dalfopristin, which are the semi-synthetic derivatives of these compounds, have already been approved for use in human medicine to treat many drug-resistant bacteria, such as methicillin-resistant staphylococci and vancomycin-resistant enterococci (Barrière et al., 1994). However, the ability of the natural producer strain *S. pristinaespiralis* ATCC25486 to synthesize pristinamycin was inefficient and required to be optimized for scale-up industrial production (Jin et al., 2010). *S. pristinaespiralis* HCCB10218, which was obtained from the random mutagenesis of the wild-type strain ATCC25486, showed significantly improved pristinamycin biosynthesis but remained insufficient for industrial production, indicating the need for further strain improvement.

With increasing knowledge of secondary metabolite biosynthetic pathways and their regulation in *Streptomyces*, rational metabolic engineering approaches that can complement the traditional "random mutagenesis and screening" method have been applied to increase the production yields of important secondary metabolites. Thus far, many different metabolic

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engineering approaches have been developed in *Streptomyces* strains, such as manipulating pathway regulation by overexpressing the genes encoding activators and/or inactivating the genes encoding repressors, increasing the supply of specific building blocks and amplifying secondary metabolite biosynthetic gene clusters of interest (Chen et al., 2010; Craney et al., 2013; Liu et al., 2013; Olano et al., 2008).

In recent years, many efforts have been made to optimize the production of important secondary metabolites by amplifying the entire biosynthetic gene clusters in *Streptomyces* strains. In fact, this strategy was developed on the basis of the observations that antibiotic-overproducing strains of *Penicillium chrysogenum*, *Streptomyces lincolnensis* and *Streptomyces kanamyceticus* (which were isolated using the traditional mutagenesis method) harbor amplifications of their respective antibiotic biosynthetic gene clusters (Fierro et al., 1995; Peschke et al., 1995; Yanai et al., 2006). For instance, the kanamycin (Km)-overproducing industrial strain *S. kanamyceticus* 12-6 contains 36 tandem copies of the Km biosynthetic gene cluster (Murakami et al., 2011a, 2011b; Yanai et al., 2006). Using this strategy, nikkomycin production in *Streptomyces ansochromogenes* and gougerotin biosynthesis in *Streptomyces graminearus* were significantly enhanced by introducing an extra copy of the nikkomycin and gougerotin biosynthetic gene clusters, respectively (Jiang et al., 2013; Liao et al., 2010).

In addition, genetic manipulation of the cluster-situated regulatory genes, which are located within the secondary metabolite biosynthetic gene clusters, is another important method to achieve titer improvements of the desired secondary metabolites in many different *Streptomyces* strains (Chen et al., 2010). For example, overexpression of the pathway-specific SARP-type activator gene *fdmR1* in a multi-copy plasmid markedly increased fredericamycin production by 5.6-fold in *Streptomyces griseus* (Chen et al., 2008). Higher titers of platensimycin and platencin were obtained in *Streptomyces platensis* by inactivating the GntR-like repressor gene *ptmR1* (Smanski et al., 2009).

Thanks to advances in our understanding of the pristinamycin biosynthetic pathways and their regulation in *S. pristinaespiralis* (Mast et al., 2011; Mast and Wohlleben, 2014), improvement of pristinamycin production through metabolic engineering approaches has become feasible. However, the co-production of PI and PII might lead to toxic effects on the producing strain as well as feedback inhibition of their own biosynthesis (Jia et al., 2006). Therefore, in this study, we only focused on improving PII production using a combined approach involving the duplication of the PII biosynthetic gene cluster and systematical engineering of seven cluster-situated regulatory genes based on the high-producing strain *S. pristinaespiralis* HCCB10218.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

The strains and plasmids used in this study are listed in Table 1. *S. pristinaespiralis* HCCB10218 (CGMCC5486) and its derivatives were grown at 30 °C on RP medium (g/L, soluble starch 20, soybean flour 10, valine 0.5, KH_2PO_4 0.5, $MgSO_4 \cdot 7H_2O$ 1, NaCl 2, $CaCO_3$ 3 and agar 20) for spore preparations. The strain HCCB10218 was obtained from the random mutagenesis of the wild-type strain ATCC25486 and cultivated in liquid RP medium (without agar) on an orbital shaker (200 rpm) at 30 °C for total DNA isolation.

Escherichia coli EPI300 and DH5α strains were used for routine DNA cloning. *E. coli* S17-1 with an integrated RP4 derivative in the bacterial chromosome was used for conjugal transfer from *E. coli* to *S. pristinaespiralis*. *E. coli* strains were grown at 37 °C in Luria-Bertani (LB) medium. Antibiotics (12.5 μg/mL chloramphenicol and 50 μg/mL apramycin) were added when necessary.

2.2. DNA manipulation

Bacterial artificial chromosome (BAC) vector pCC1BAC (Epicentre) was isolated and purified using an E.Z.N.A.® BAC/PAC DNA Kit (Omega Bio-Tek). PCR products and restrictive enzymatic digestion products were purified using an AxyPrep DNA Gel Extraction Kit or an Agarose Gel DNA Extraction Kit (Roche). The isolation of the *S. pristinaespiralis* genomic DNA and intergeneric conjugal transfer were performed as described by Kieser et al. (2000).

Classical and modified Gibson DNA assembly methods were performed similarly as described previously (Gibson et al., 2008, 2009). In brief, 5 μL of equimolar DNA (including the overlapping DNA fragments and linearized BAC vector) was added to 15 μL of the Gibson assembly master mixture, which was prepared as follows (a total volume of 1200 μL): 320 μL of 5 × isothermal (ISO) reaction buffer, 0.64 μL of 10 U/μL T5 exonucleases (Epicentre), 20 μL of 2 U/μL Phusion polymerase (NEB), 160 μL of 40 U/μL Taq ligase (NEB) and 700 μL of double distilled water. Normally, 80 ng of the linearized pCC1BAC vector (8 kb) was used. The reaction mixture was incubated at 50 °C for 1 h. Subsequently, 1 μL of the assembly product was transferred into *E. coli* EPI300 by electroporation. The cells were recovered for 2 h in 1 mL of LB medium and then incubated on LB agar containing 12.5 μg/mL chloramphenicol at 37 °C for 24–36 h. The linearized pCC1BAC vector was prepared by PCR amplification using the corresponding primers listed in Table S1, followed by digestion with *Dpn*I to remove the circular template.

The PII biosynthetic gene cluster was designed to contain 14 overlapping DNA fragments (F1 to F14) (Fig. 1A). The F15 fragment, which contained the apramycin resistance *aac(3)IV* cassette, the *phiC31* integrase expression cassette, an *oriT* (RK2) site and an *attP* site (cloned from the integrative vector pSET152, Table 1), was also included. The entire assembly process was organized into three hierarchical levels. Briefly, 15 initial DNA fragments (from F1 to F15, with approximately 4–5 kb each in length) with corresponding end overlaps (containing the introduced restriction enzyme sites) (Table S1) were obtained by PCR amplification using a high-fidelity DNA polymerase (KOD-Plus-Neo, TOYOBO) and assembled in triplets in the first level. The intermediate fragments, including F1–F3, F4–F6 and F7–F9 (approximately 15 kb each in length; left, middle and right), which were created by digesting with the appropriate restriction enzymes, were further assembled in the second level into two larger constructs, F1–F9 and F10–F15 (approximately 45 kb and 27 kb in length, respectively). Finally, the above two constructs were ligated together to yield the final product BAC-F1F15 in the third level (Fig. 1B and C). The recombinant vector BAC-F15, which only carried the F15 fragment, was also constructed using our modified Gibson assembly method.

2.3. Construction of the plasmids and S. pristinaespiralis strains

The plasmids pIB-*spbR*, pIB-*papR1*, pIB-*papR2*, pIB-*papR4* and pIB-*papR6* were constructed as follows. The respective gene sequences of *spbR*, *papR1*, *papR2*, *papR4* and *papR6* were obtained by PCR using the corresponding primers listed in Table S1. Each PCR amplicon was cloned into an integrative vector pIB139 between *Nde*I and *Eco*RI. To construct the pIB-*pap*R4R6 plasmid, the individual gene sequence of *papR4* and *papR6* was amplified from the genomic DNA of HCCB10218 using the primer pairs OE-*papR4*-fw(R4R6)/rev(R4R6) and OE-*papR6*-fw(R4R6)/rev(R4R6), respectively (Table S1). After digestion with *Nde*I/*Bgl*II and *Bgl*II/*Eco*RI, respectively, the resulting products were cloned simultaneously into pIB139 between *Nde*I and *Eco*RI. All of the constructed vectors were confirmed by DNA sequencing. The above mentioned plasmids were introduced individually into the parental strain *S. pristinaespiralis* HCCB10218 or its derivatives through conjugal transfer, generating the corresponding overexpression strains listed in Table 1. The parental strain HCCB10218 with pIB139 (10218/pIB139) was used as a negative control.

Table 1
Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant features	Source
E. coli		
DH5α	F$^-$ 80Φ*dlacZ*DM15Δ(*lacZYA*−*argF*)*U169deoR recA1 endA1 hsdR17*(rk$^-$ mk$^+$) *supE44* λ$^-$ *thi*$^-$*1gyrA96 relA1*	GIBCO-BRL
EPI300	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*dlacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara*, *leu*)*7697 galU galK* λ- *rpsL* (*StrR*) *nupG trfA tonA*	Epicentre
S17-1	*supE44*,Δ*lacU169* (Φ*lacZ*ΔM15), *recA1*, *endA1*, *hsdR17*, *thi-1*, *gyrA96*, *relA1*, *par* phage lysogenic	GIBCO-BRL
B. subtilis		
ATCC6633	The indicator strain for the bioassay	CGMCC
S. pristinaespiralis		
HCCB10218	The parental strain derived from the wild-type *S. pristinaespiralis* ATCC25486	CGMCC
10218/BAC-F15	HCCB10218 containing plasmid BAC-F15	This study
10218/BAC-F1F15	HCCB10218 containing plasmid BAC-F1F15	This study
ΔPII	Mutant with in-frame deletion of most of the PII biosynthetic gene cluster (from 42,410 bp to 96,760 bp in the GenBank: FR682001.1)	This study
ΔPII/BAC-F15	ΔPII containing the BAC-F15 plasmid	This study
ΔPII/BAC-F1F15	ΔPII containing the BAC-F1F15 plasmid	This study
Δ*papR3*	Mutant with deletion of the *papR3* gene	This study
Δ*papR5*	Mutant with deletion of the *papR5* gene	This study
Δ*papR3-R5*	Mutant with deletion of both *papR3* and *papR5*	This study
Δ*papR3*/BAC-F15	Δ*papR3* carrying the BAC-F15 plasmid	This study
Δ*papR5*/BAC-F15	Δ*papR5* carrying the BAC-F15 plasmid	This study
Δ*papR3*/BAC-F1F15	Δ*papR3* carrying the BAC-F1F15 plasmid	This study
Δ*papR5*/BAC-F1F15	Δ*papR5* carrying the BAC-F1F15 plasmid	This study
10218/pIB139	HCCB10218 carrying the pIB139 plasmid	This study
10218/pIB-*papR1*	HCCB10218 carrying the pIB-*papR1* plasmid	This study
10218/pIB-*papR2*	HCCB10218 carrying the pIB-*papR2* plasmid	This study
10218/pIB-*spbR*	HCCB10218 carrying the pIB-*spbR* plasmid	This study
10218/pIB-*papR4*	HCCB10218 carrying the pIB-*papR4* plasmid	This study
10218/pIB-*papR6*	HCCB10218 carrying the pIB-*papR4* plasmid	This study
10218/pIB-*papR4R6*	HCCB10218 carrying the pIB-*papR4R6* plasmid	This study
Δ*papR3*/pIB139	Δ*papR3* carrying the pIB139 plasmid	This study
Δ*papR5*/pIB139	Δ*papR5* carrying the pIB139 plasmid	This study
Δ*papR3*/pIB-*papR4R6*	Δ*papR3* carrying the pIB-*papR4R6* plasmid	This study
Δ*papR5*/pIB-*papR4R6*	Δ*papR5* carrying the pIB-*papR4R6* plasmid	This study
Δ*papR3*+*R4R6*	Δ*papR3* carrying the integrative expression cassette of *papR4* and *papR6* without an antibiotic-resistant marker	This study
Δ*papR5*+*R4R6*	Δ*papR5* carrying the integrative expression cassette of *papR4* and *papR6* without an antibiotic-resistant marker	This study
Δ*papR5*+*R4R6*/BAC-F15	Δ*papR5*+*R4R6* carrying the BAC-F15 plasmid	This study
Δ*papR5*+*R4R6*/BAC-F1F15	Δ*papR5*+*R4R6* carrying the BAC-F1F15 plasmid	This study
Δ*papR3*+*R4R6*/BAC-F15	Δ*papR3*+*R4R6* carrying the BAC-F15 plasmid	This study
Δ*papR3*+*R4R6*/BAC-F1F15	Δ*papR3*+*R4R6* carrying the BAC-F1F15 plasmid	This study
Plasmids		
pCC1BAC	A BAC plasmid derived from pBeloBAC11 and pIndigoBAC-5 containing a single copy *E. coli* F-factor replicon and a high-copy origin of replication called "*oriV*"	Epicentre
pSET152	pUC19 ori, *ΦC31 int/attP*, *aac(3)IV*, *lacZα*, and *oriT* RK2	Bierman et al. (1992)
pKC1139	A replicative vector in actinomycetes harboring a temperature sensitive replicon pSG5, *oriT*, and *aac(3)IV*	Kieser et al. (2000)
pIB139	An integrative plasmid containing *oriT*, *attP*, *int*, *aac(3)IV* and *ermE**p	Kieser et al. (2000)
pMD18-T	A simple TA cloning vector derived from the pUC18 vector	Takara
pALSceI	An *E. coli-Streptomyces* shuttle-replicating plasmid containing I-SceI under the control of the *tipA* promoter, *oriT*, and a hygromycin-resistant gene	This study
BAC-F15	A BAC plasmid derived from pCC1BAC, with the fragment F15 containing all integrative and screening parts (*ΦC31 int/attP*, *aac(3)IV*, and *oriT*) fom pSET152	This study
BAC-F1F15	A pCC1BAC plasmid carrying the *in vitro* assembled pristinamycin II biosynthetic gene cluster	This study
pKC1139-PII	pKC1139 with two homologous arms containing the upstream and downstream DNA fragments of the deleted PII biosynthetic genes	This study
pKC1139-*papR3*	pKC1139 with two homologous arms containing the upstream and downstream DNA fragments of *papR3*	This study
pKC1139-*papR5*	pKC1139 with two homologous arms containing the upstream and downstream DNA fragments of *papR5*	This study
pKC1139-2344/5/*R4R6*	pKC1139 with two homologous arms containing the upstream and downstream DNA fragments of the genes SSDG_02344/5 and the *papR4*/*papR6* expression cassette	This study
pIB-*papR1*	pIB139 with *papR1* expression under the control of the strong constitutive promoter *ermE**p	This study
pIB-*papR2*	pIB139 with *papR2* expression under the control of the strong constitutive promoter *ermE**p	This study
pIB-*spbR*	pIB139 with *spbR* expression under the control of the strong constitutive promoter *ermE**p	This study
pIB-*papR4*	pIB139 with *papR4* expression under the control of the strong constitutive promoter *ermE**p	This study
pIB-*papR6*	pIB139 with *papR6* expression under the control of the strong constitutive promoter *ermE**p	This study
pIB-*papR4R6*	pIB139 with the expression of both *papR4* and *papR6* under the control of the strong constitutive promoter *ermE**p	This study

The mutant ΔPII with an in-frame partial deletion of the PII biosynthetic gene cluster (from 42,410 bp to 96,760 bp; GenBank accession no.: FR682001.1) was constructed using the I-*Sce*I endonuclease-mediated markerless deletion method described by Lu et al. (2010) with some modifications. Briefly, two homologous arms (1164 and 1169 bp in length) containing the upstream and downstream regions of the target PII biosynthetic genes were amplified from the HCCB10218 genomic DNA using the primer pairs DE-PII-up-fw/rev and DE-PII-down-fw/rev (the primer DE-PII-down-rev contains the cleavage site of I-*Sce*I) (Table S1), followed by treatment with *Hin*dIII/*Xba*I and *Xba*I/*Eco*RI, respectively. The resulting DNA fragments were cloned simultaneously into a temperature-sensitive plasmid pKC1139 between *Hin*dIII and *Eco*RI to yield pKC1139-PII (Table 1). The obtained plasmid was integrated into the HCCB10218 chromosome by conjugal transfer on solid RP medium supplemented with 50 μg/mL apramycin at 37 °C, resulting in the single cross-over strain 10218/pKC1139-PII. Subsequently, the pALSceI plasmid, which contains the codon-optimized I-*Sce*I gene under the control of the thiostrepton-inducible promoter *tipA* (Table 1), was transferred into 10218/pKC1139-PII. The expression of I-*Sce*I was induced by thiostrepton to accelerate the occurrence of double cross-overs in liquid RP medium at 30 °C. Finally, apramycin-sensitive strains were selected from thiostrepton-resistant colonies and verified by PCR using the primers ID-PII-fw/rev (Fig. S1A), followed by DNA sequencing. The correct double-crossover strain was grown on RP medium without thiostrepton for three rounds to remove the pALSceI plasmid, generating the mutant ΔPII.

The strains Δ*papR3*, Δ*papR5*, Δ*papR3-R5*, Δ*papR3*+*R4R6* and Δ*papR5*+*R4R6* were constructed by using the standard procedure with the temperature-sensitive plasmid pKC1139 (Kieser et al., 2000). To construct the mutant Δ*papR3* with an in-frame deletion of the gene *papR*3 sequence, two homologous arms (1229 and 1243 bp in length) containing the upstream and downstream regions of *papR3* were obtained by PCR using the primer pairs DE-*papR3*-up-fw/rev and DE-*papR3*-down-fw/rev, and double-digested with *Hin*dIII/*Xba*I and *Xba*I/*Eco*RI, respectively. The resulting DNA fragments were cloned into pKC1139 between *Hin*dIII and *Eco*RI to yield pKC1139-*papR3* (Table 1). The obtained plasmid was integrated into the HCCB10218 chromosome by conjugal transfer on solid RP medium supplemented with 50 μg/mL apramycin at 37 °C, resulting in the single cross-over strain 10218/pKC1139-*papR3*. The resultant strain was subsequently grown in liquid RP medium without apramycin at 30 °C for three rounds. The correct double cross-overs were verified by PCR using the primers ID-*papR3*-fw/rev, yielding the mutant Δ*papR3* (Fig. S1B). The strains Δ*papR5* (with an in-frame deletion of the *papR5* gene) and Δ*papR3-R5* (with an in-frame deletion of the *papR5* gene based on Δ*papR3*) were constructed using HCCB10218 and Δ*papR3* as the starting strains, respectively, by the same approach as described above. The primers used are listed in Table S1.

Similarly, we constructed the mutant Δ*papR3*+*R4R6*, which has the expression cassette of both *papR4* and *papR6* integrated into the genome of Δ*papR3*. Briefly, two homologous arms (1513 and 1560 bp in length) flanking *SSDG-02344/5* were amplified by PCR using the corresponding primer pairs DE-*2344/5*-up-fw/rev and DE-*2344/5*-down-fw/rev. The PCR products were double-digested with *Hin*dIII/*Xba*I and *Xba*I/*Eco*RV, respectively, and cloned into pKC1139 between *Hin*dIII and *Eco*RV to yield pKC1139-*2344/5*. The *papR4-R6* expression cassette was amplified from the plB-*papR4R6* plasmid using the primers *papR4R6*-fw/rev (Table S1). In this cassette, *papR4* and *papR6* expression was under the control of the strong constitutive promoter *ermE**p. After *Xba*I treatment, the resulting fragment was cloned into pKC1139-*2344/5* to yield pKC1139-*2344/5/R4R6*. The correct strain Δ*papR3*+*R4R6* was verified by PCR using the primers ID-2344/5-fw/rev (Fig. S1C), followed by DNA sequencing. Using the same approach, the strain Δ*papR5*+*R4R6* was constructed by integrating the *papR4-R6* expression cassette into the Δ*papR5* genome. The primers used are listed in Table S1.

2.4. Fermentation of S. pristinaespiralis, microscopic observation of bacterial growth and analysis of pristinamycin production

S. pristinaespiralis strains were grown on solid RP medium at 30 °C for 4–5 days and then inoculated into seed medium (g/L, soluble starch 15, glucose 10, soybean flour 15, peptone 5, yeast extract 5, KNO_3 2.5, NaCl 2 and $CaCO_3$ 4). The pH value was adjusted to 7.0–7.2 prior to $CaCO_3$ addition and sterilization. For *S. pristinaespiralis* fermentation in the Erlenmeyer flask, the cultures were incubated at 27 °C in 25 mL of seed medium in 250 mL Erlenmeyer flasks on an orbital shaker (240 rpm). After 44–48 h, 2 mL of pre-cultures was inoculated into 25 mL of fermentation medium (g/L, soluble starch 40, glucose 22.5, cotton seed meal 32, yeast extract 3.5, KH_2PO_4 0.1, $MgSO_4 \cdot 7H_2O$ 0.5 and $CaCO_3$ 6). The pH was adjusted to 6.0 prior to $CaCO_3$ addition and sterilization. When necessary, 6% of the macroreticular resin HB60 (Shanghai Minyong Enterprise Co., Ltd.) was added after the fermentation cultures had been cultivated for 24 h. The resin was sterilized separately at 121 °C for 30 min.

For the batch fermentation with resin in a bioreactor, the strains were grown at 27 °C in 75 mL of seed medium in 500 mL Erlenmeyer flasks on an orbital shaker (240 rpm). After 44–48 h, 300 mL of seed cultures (from four flasks) was added to 4 L of fermentation medium in a 5-L BioFlo 110 Fermentor (New Brunswick Scientific) at 27 °C with a stirring speed of 550 rev/min and an aeration rate of 1 vvm. Sterilized HB60 resin was added after the fermentation cultures had been cultivated for 24 h.

Crystal violet staining was used for the morphological observation of *S. pristinaespiralis* strains grown in the fermentation medium. Briefly, fermentation samples were collected at 48 and 72 h. A small amount of each sample was coated on the glass slides and examined after routine staining with 1% crystal violet by a light microscope.

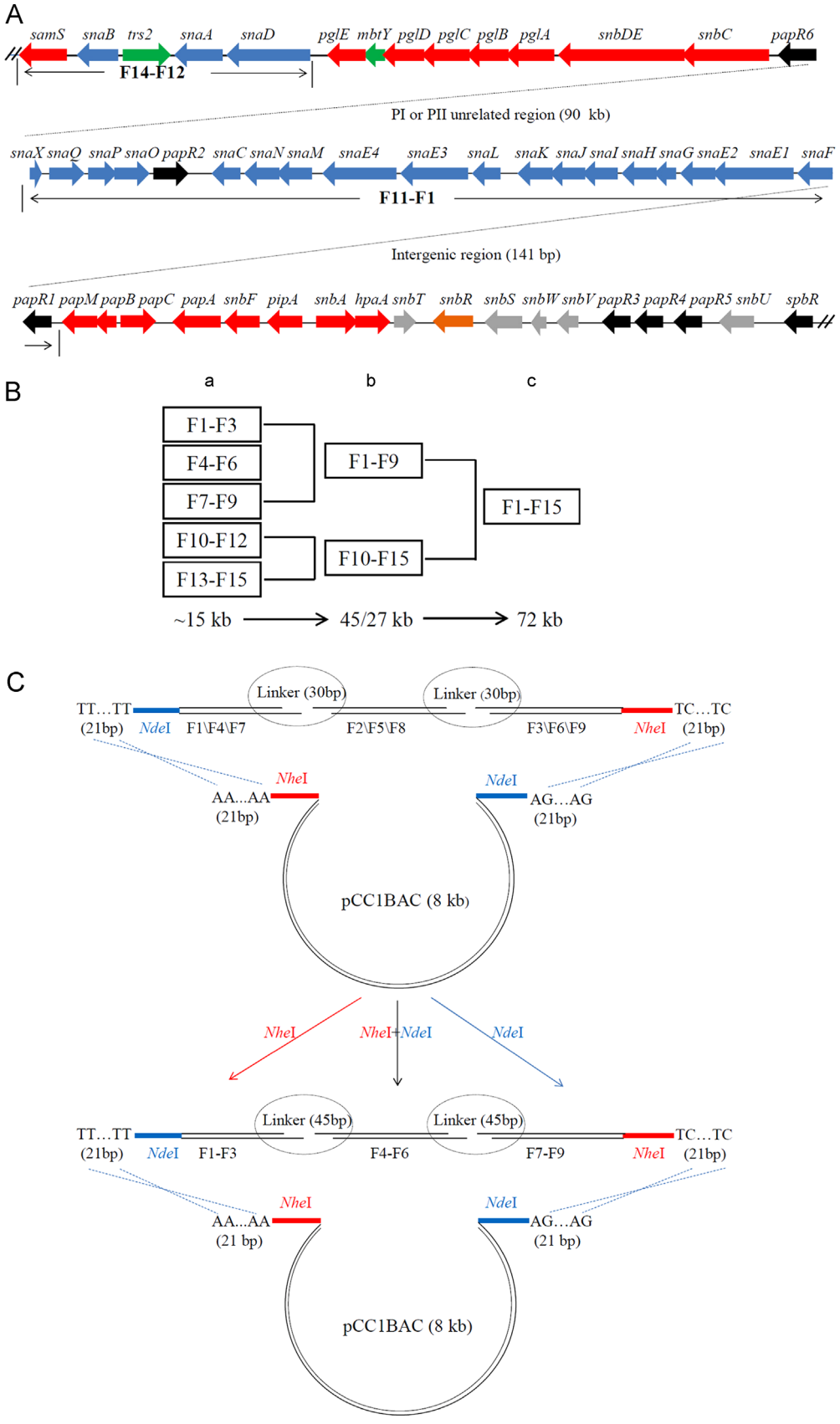
For analysis of pristinamycin production, fermentation samples were collected at seven time points (30, 48, 60, 72, 84, 96 and 120 h) and extracted with the same volume of acetone for 2 h. The mixtures were centrifuged at 12,000 rpm for 10 min and the supernatants were collected. A paper disk diffusion bioassay was performed to determine the bactericidal capacity of pristinamycin, and *Bacillus subtilis* ATCC6633 was used as an indicator strain. Briefly, *B. subtilis* was grown in liquid LB medium at 37 °C for 4–6 h. An aliquot (50 μL) of the culture was mixed with 7–8 mL LB medium containing 1% low melting point agarose (Shanghai Sangon Biotech Co., Ltd.) and then spread on solid LB agar plates. Sterile filter paper discs (6 mm in diameter) were placed onto the plates and impregnated with the supernatants (10 μL) collected at 48 h after fermentation. The plates were incubated at 37 °C for 16 h to observe the inhibition zones. For quantitative analysis, the supernatants (acetone extracts) were directly analyzed by HPLC (1100 series, Agilent) using a 4.6 × 150 mm Zorbax Eclipse XDB-C18 column (Agilent). For HPLC detection, a mixture of acetonitrile/0.03 M KH_2PO_4 (45:55, v/v, pH of KH_2PO_4 was 2.75) was used as the mobile phase, with a flow rate of 1.0 mL/min and a retention time of 10 min. The elute was monitored at 206 nm, and the column temperature was 30 °C. The chromatograms of PI_A and PII_A (the main components of PI and PII, respectively) are presented in Fig. S2A. Production of PI_A and PII_A was calculated from the standard curves (Fig. S2B).

2.5. RNA preparation and real-time RT-PCR (qRT-PCR)

S. pristinaespiralis samples were collected from the fermentation medium at different time points and frozen immediately in liquid nitrogen. RNA samples were prepared using an Ultrapure RNA Kit (CWBIO). Subsequently, RNA preparations were digested

with DNase I (Takara) to remove contaminating chromosomal DNA. A reverse transcriptase kit (Invitrogen) and random hexamers (Takara) were used for the reverse transcription of total RNA.

qRT-PCR analysis was performed using a previously described method (Wang et al., 2013). The primers used are listed in Table S1. The *hrdB* gene (*SSDG_06142*, encoding the RNA polymerase



principal sigma factor) was used as an internal control. qRT-PCR was performed in triplicate for each transcript and repeated with three independent RNA samples. The relative expression levels of the tested genes were normalized to *hrdB*. The relative fold changes in the expression of each gene were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The error bars indicate the standard deviations from three independent biological replicates. The data were analyzed by Student's *t*-test, with $^{*}p < 0.05$ and $^{**}p < 0.01$ indicating significant differences.

3. Results

3.1. Modification of the classical Gibson assembly method for cloning large DNA fragments with high GC contents

Amplification of secondary metabolite biosynthetic gene clusters has been proved to be an efficient approach for improving the production of desired secondary metabolites in actinomycetes (Murakami et al., 2011a, 2011b). Herein, we aimed to enhance PII production of *S. pristinaespiralis* HCCB10218 by adding an extra copy of the PII biosynthetic gene cluster. In order to clone the discrete PII biosynthetic gene cluster (Fig. 1A), the classical Gibson isothermal, one-step assembly method was employed (Gibson et al., 2009). The entire gene cluster was designed into 14 DNA fragments that were 4–5 kb each in length, and the assembly process was organized into three hierarchical levels (Fig. 1B and C). The F15 fragment, which contains the apramycin resistance *aac(3)IV* cassette, the *phiC31* integrase expression cassette, an *oriT* (RK2) site and an *attP* site (cloned from the integrative vector pSET152), was also included, enabling the integration of the assembled PII biosynthetic gene cluster into the *S. pristinaespiralis* chromosome by conjugal transfer.

We tested the Gibson assembly method with two groups of three 5 kb DNA fragments (F7–F9 and F10–F12, Fig. 2A). For each group, 20 colonies were randomly picked and cultivated for BAC vector isolation to identify the correct DNA assembly by using *Nde*I digestion. The experiments were repeated twice. Unfortunately, only one BAC construct overall (1/80) was correct after all four parallel attempts, and most of the constructs were generated by the self-ligation of the BAC vector (Fig. 2A and Table 2). These results indicated that the classical Gibson assembly method was unsuitable for cloning the PII biosynthetic gene cluster. We reasoned that the high BAC self-ligation rate might be primarily because of the high GC ($>75\%$) end overlaps (40 bp) in two sides of the vector backbone. In the Gibson reaction system, the low isothermal condition (50 °C) was previously proposed to enable the overlaps with high GC contents to easily form mismatched linker pairings (Casini et al., 2014).

To address this issue, we modified the approach using the following two strategies. First, two universal terminal single-stranded DNA overhangs with high AT contents were added to the ends of the BAC vector, which may decrease vector self-ligation (Fig. 1C). This novel design made the linearized BAC vector eligible for repeated use in independent assemblies. In the classical Gibson method, different linearized BAC vectors had to be prepared in each reaction for assembling different DNA fragments. Second, we introduced two restriction enzyme sites (*Nde*I/*Nhe*I) into the respective sides of the designed overhangs to achieve the hierarchical and seamless assembly of large DNA molecules. Thus, the left, middle and right assembled fragments in the next assembly process could be obtained by digestion with *Nhe*I, *Nhe*I/*Nde*I and *Nde*I, respectively (Fig. 1C).

We chose the same two groups of DNA fragments used above, F7–F9 and F10–F12, to test the efficacy of the modified Gibson assembly method. The results showed that adding the universal AT-rich vector end overhangs resulted in a marked increase in the assembly efficiency from 0% (0/20) to 30% (6/20) and a significant decrease in the vector self-ligation ratio from 80% to 45% compared with the classical method (Fig. 2B and Table 2). In the second level, the larger DNA molecule, F1–F9 (45 kb), was assembled with an assembly efficiency of 20% (4/20) (Fig. 2C). These results suggested that the modified Gibson assembly method could be efficiently applied to clone large DNA fragments with high GC contents.

3.2. In vitro assembly of the entire PII biosynthetic gene cluster

In the present study, the improved Gibson assembly method was used to clone the entire PII biosynthetic gene cluster, which spans 67 kb. Unfortunately, we failed to obtain the correct DNA construct (BAC-F1F15) during the last assembly process (from 27 and 45 kb to 72 kb). To address this issue, we extended the overlaps between the DNA inserts and BAC vector from 21 bp to 40 bp and successfully obtained the PII biosynthetic gene cluster (BAC-F1F15) with an efficiency of 40% (8/20) (Fig. 2D). The construct BAC-F1F15 was further verified by restriction analysis using another three restriction enzymes (Fig. 3A), followed by 454 sequencing to confirm whether the assembled gene cluster was correct. DNA sequencing revealed that, totally, eight point mutations (including two synonymous mutations, two point mutations in the noncoding regions and four nonsynonymous mutations) and two deletions in the F6/F8 fragments were introduced by PCR (Table S1). To repair these two deletions, we divided F6 and F8 into two smaller fragments, F6-1\F6-2 and F8-1\F8-2, respectively. These four smaller fragments were obtained by PCR amplification and cloned into the pMD18-T vector for DNA sequencing. The correct DNA fragments were assembled into F6 and F8 using the modified method. Finally, we re-assembled the target gene cluster (BAC-F1F15). It should be noted that the point mutations introduced by PCR in the assembled gene cluster were not repaired.

To determine whether the assembled gene cluster (BAC-F1F15) could exert its function *in vivo*, a complementary genetic analysis was performed. First, a ΔPII mutant with the deletion of a large part (54 kb/67 kb) of the PII biosynthetic gene cluster was constructed using the I-*Sce*I endonuclease-mediated method, and the correct mutant was verified by PCR (Fig. S1A). Subsequently, BAC-F1F15 was introduced into ΔPII, generating the complemented strain ΔPII/BAC-F1F15. ΔPII with the introduction of BAC-F15 was also constructed, resulting in ΔPII/BAC-F15. Fermentation samples of four *S. pristinaespiralis* strains, including the parental strain HCCB10218, ΔPII, ΔPII/BAC-F15 and ΔPII/BAC-F1F15, were collected at 48 h, and pristinamycin production was analyzed by HPLC (Fig. 3B). Intriguingly, no production of PI_A or PII_A was observed in ΔPII and ΔPII/BAC-F15. A SARP-type regulatory gene *papR2*, which

Fig. 1. The hierarchical assembly strategy for cloning the PII biosynthetic gene cluster using the modified Gibson assembly method. (A) The gene organization of the pristinamycin biosynthetic gene cluster. Pristinamycin II (PII) and I (PI) biosynthetic genes are indicated with blue and red arrows, respectively. Black and yellow arrows represent the regulatory genes (from *papR1* to *papR6* and *spbR*) and the *snbR* gene (encoding a predicted ABC transporter that likely confers resistance to pristinamycin), respectively. Gray arrows represent the genes of unknown function (including *snbT*, *snbS*, *snbW*, *snbV* and *snbU*). The PII biosynthetic genes to be assembled *in vitro*, including the *samS* gene, were split into 14 fragments (F1 to F14). (B) The hierarchical assembly pattern of the PII biosynthetic gene cluster. The F15 fragment, which contains the apramycin resistance *aac(3)IV* cassette, the *phiC31* integrase expression cassette, an *oriT* (RK2) site and an *attP* site, was cloned from the integrative vector pSET152. (C) The pattern of the first and second levels of assembly. The restriction enzyme sites (*Nde*I and *Nhe*I) and the universal overlaps of all DNA fragments (including the linearized BAC vector) were introduced by PCR amplification. In the first level, three DNA fragments were assembled simultaneously using the vector pCC1BAC. The overlaps between two DNA inserts are 30 bp in length. In the second level, the left, middle and right assembled products (F1–F3, F4–F6 and F7–F9, respectively) from the first level were digested for the next level of assembly by *Nhe*I, *Nhe*I/*Nde*I and *Nde*I, respectively. The overlaps between two DNA inserts are 45 bp at the second level. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

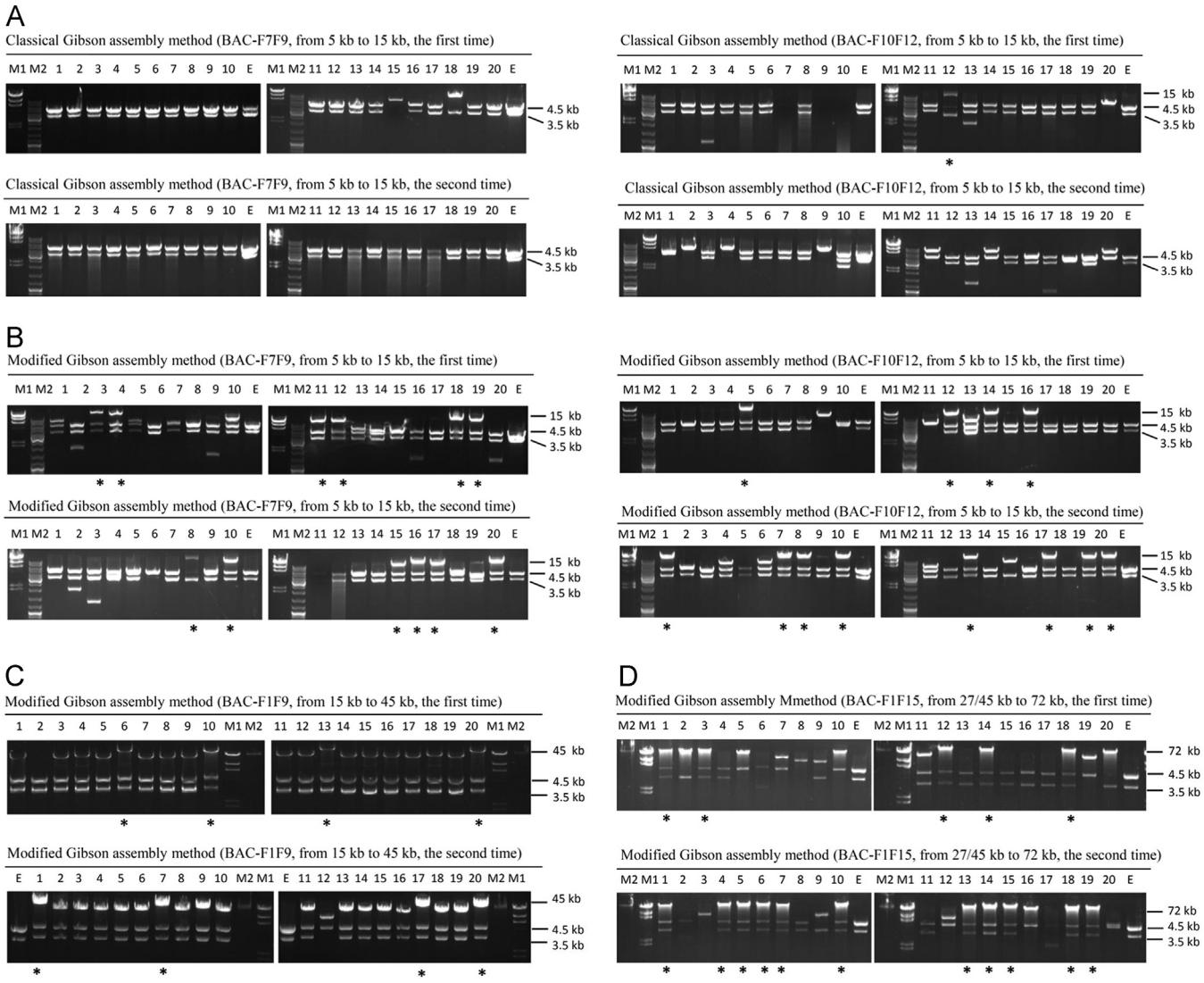


Fig. 2. Assembly efficiencies of the modified and classical Gibson assembly methods. *Nde*I restriction analysis of the recombinant BAC plasmids that were isolated from 20 randomly picked clones of the first assembly level (from 5 kb to 15 kb) for the classical Gibson assembly method (A) and the modified Gibson assembly method (B). The analysis was repeated twice with two groups of three different fragments (F7–F9 and F10–F12) from the PII biosynthetic gene cluster. The expected band sizes after *Nde*I digestion were 3.5, 4.5 and 15 kb. M1 and M2 represent the 1 kb DNA ladder (Thermo Scientific) and λ *Hin*dIII DNA ladder (Takara), respectively. *Nde*I restriction analysis of the BAC plasmids prepared from 20 independent random clones of the second-level assembly from 15 kb to 45 kb (C) and the third-level assembly from 27 kb/45 kb to 72 kb (D) for the modified Gibson assembly method. The analysis was repeated twice. M1 and M2 represent the λ *Hin*dIII DNA ladder and λ DNA (NEB), respectively. E represents the pCC1BAC control vector with universal overlaps. Asterisk (*) indicates the correct assembly.

Table 2
A comparison of assembly efficiencies for the classical and modified Gibson assembly methods. The data in the brackets represent two replicates. ND: not detected.

DNA size	From 5 kb to 15 kb		From 15 kb to 45 kb	From 27/45 kb to 72 kb
Assembled fragments	BAC-F7F9	BAC-F10F12	BAC-F1F9	BAC-F1F15
Modified method	30% (6/20+6/20)	30% (4/20+8/20)	20% (4/20+4/20)	40% (5/20+11/20)
Classical method	0 (0/20+0/20)	2.5% (1/20+0/20)	ND	ND

is situated in the PII biosynthetic gene cluster, is essential for both PI and PII biosynthesis (data not shown), thus deletion of the PII gene cluster led also to loss of PI production. Introducing BAC-F1F15 into ΔPII could partially recover the production of both PI_A and PII_A (Fig. 3B), clearly revealing that the assembled gene cluster was functional *in vivo*. The complemented strain ΔPII/BAC-F1F15 did not produce as much PII_A as the parental strain HCCB10218. This might be mainly due to the point mutations introduced by PCR and/or the different integrated location of the assembled PII biosynthetic gene cluster in the genome.

3.3. Improvement of PII production by integrating the assembled PII biosynthetic gene cluster into the genome of *S. pristinaespiralis* HCCB10218

To improve PII production by cluster amplification, we introduced the assembled gene cluster (BAC-F1F15) into the high-producing parental strain *S. pristinaespiralis* HCCB10218. In parallel, HCCB10218 and its derivative 10218/BAC-F15 (with the integration of BAC-F15 into the HCCB10218 genome) were used as the negative controls. Fermentation samples of the *S. pristinaespiralis* strains were collected

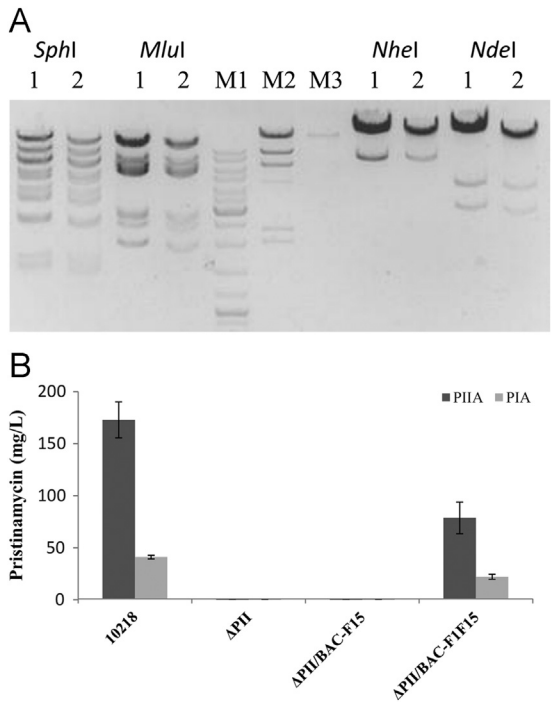


Fig. 3. Restriction and functional analyses of the assembled PII biosynthetic gene cluster. (A) Restriction analysis of the assembled PII biosynthetic gene cluster (BAC-F1F15) in a 1% agarose gel. The expected band sizes of BAC-F1F15, which was digested with four restriction endonucleases, were as follows: 4 × 1.5, 2 × 2.6, 3.4, 2 × 3.7, 4.1, 5, 5.7, 2 × 7.7, 10.6 and 17.8 kb (*Sph*I); 2, 2.7, 2.9, 3.1, 3.5, 2 × 6.1, 6.6, 7.1, 8.3, 14.6 and 17.9 kb (*Mlu*I); 8 and 72 kb (*Nhe*I); and 3.5, 4.5 and 72 kb (*Nde*I). M1, M2 and M3 represent the 1 kb DNA ladder, λ *Hin*dIII DNA ladder and λ DNA, respectively. (B) Pristinamycin production by the strains ΔPII and ΔPII/BAC-F1F15. The parental strain HCCB10218 and ΔPII/BAC-F15 were used as the positive and negative controls, respectively. Fermentation samples were taken at 48 h, and PII_A production was analyzed by HPLC. Fermentations were performed in triplicates.

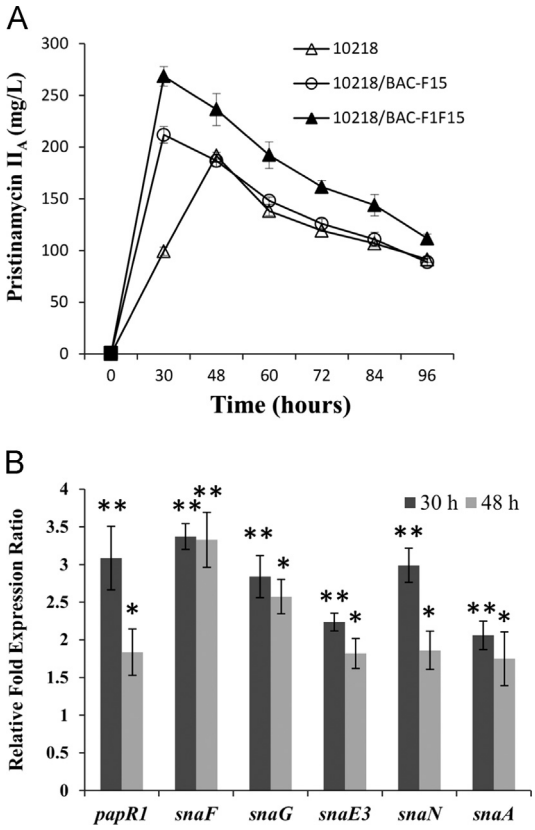


Fig. 4. The effects of duplicating the assembled PII biosynthetic gene cluster in the parental strain HCCB10218 on PII_A production and gene expression. (A) PII_A production profiles of 10218/BAC-F1F15 compared with HCCB10218 and 10218/BAC-F15. Fermentation samples were collected at the six indicated time points, and fermentations were performed in triplicates. (B) Transcriptional analysis of six selected PII biosynthetic genes in 10218/BAC-F1F15 compared with 10218/BAC-F15. Fermentation samples for RNA isolation were collected at 30 and 48 h. The *hrdB* gene was used as an internal control. The relative transcription levels of the tested genes were normalized to the *hrdB* gene. The relative fold changes in the expression of each gene (10218/BAC-F115 *versus* 10218/BAC-F15) were determined using the $2^{-\Delta\Delta Ct}$ method. The error bars indicate the standard deviations from three independent biological replicates, $^{*}p < 0.05$ and $^{**}p < 0.01$.

at six time points (30, 48, 60, 72, 84 and 96 h) and their PII_A production was analyzed by HPLC. The results showed that introduction of BAC-F1F15 (10218/BAC-F1F15) resulted in a maximum increase in PII_A biosynthesis by 45% (from 185 mg/L to 268 mg/L, Fig. 4A). 10218/BAC-F15 exhibited a slight improvement in PII_A production over that of HCCB10218 (from 185 mg/L to 209 mg/L, Fig. 4A), possibly because of an unknown vector effect.

To examine the effects of duplicating the PII biosynthetic gene cluster at the transcriptional level, we performed real-time RT-PCR (qRT-PCR) analysis. Six PII biosynthetic genes, including *papR1*, *snaF*, *snaG*, *snaE3*, *snaN* and *snaA*, were selected and tested. RNA samples were isolated from the fermentation cultures of 10218/BAC-F15 and 10218/BAC-F1F15 at 30 and 48 h. It is observed that the expression levels of all the tested genes were enhanced at least two-fold upon gene cluster duplication (Fig. 4B). These results suggested that amplification of the PII biosynthetic gene cluster in the parental strain HCCB10218 could increase the transcription of the PII biosynthetic genes and then facilitate PII biosynthesis.

3.4. Systematical engineering of the cluster-situated regulatory genes to improve PII production

As described above, introduction of an extra copy of the PII biosynthetic gene cluster into the parental strain HCCB10218 led to a limited improvement of PII production (from 185 to 268 mg/L). We attempted to combine other metabolic engineering strategies for constructing a higher PII-producing strain. Seven regulatory genes are situated within the pristinamycin biosynthetic cluster, which may form a strict, complex regulatory system (Mast et al., 2011; Mast and Wohlleben, 2014) and possibly account for the small improvement of PII biosynthesis after cluster duplication. Therefore, systematical engineering of the cluster-situated regulatory genes was also performed in this study.

3.4.1. Deleting the repressor genes

papR3 and *papR5*, which encode two TetR-family regulators and have been identified to function as repressors of pristinamycin biosynthesis (Mast et al., 2011), were deleted individually. Two deletion mutants Δ*papR3* and Δ*papR5* were obtained, which were confirmed by PCR analysis and DNA sequencing (Fig. S1B). HPLC analysis revealed that inactivation of either *papR3* or *papR5* resulted in significantly enhanced PII_A production along the tested time course (Fig. 5A). Δ*papR3* and Δ*papR5* showed maximum increases in PII_A biosynthesis by 59% and 66%, respectively, in comparison to the parental strain (Fig. 5A). It is worth noting that Δ*papR3* showed a PII_A production curve similar to that of the parental strain, which reached the maximum PII_A level at 48 h, followed by a quick decline, whereas Δ*papR5* produced the maximum PII_A level at approximately 60 h and maintained this high level until 84 h (Fig. 5A). The distinct PII production profiles were potentially due to the different regulatory modes of these two TetR-family repressors in the regulation of PII biosynthesis. In addition, the growth analysis by the light microscope revealed that

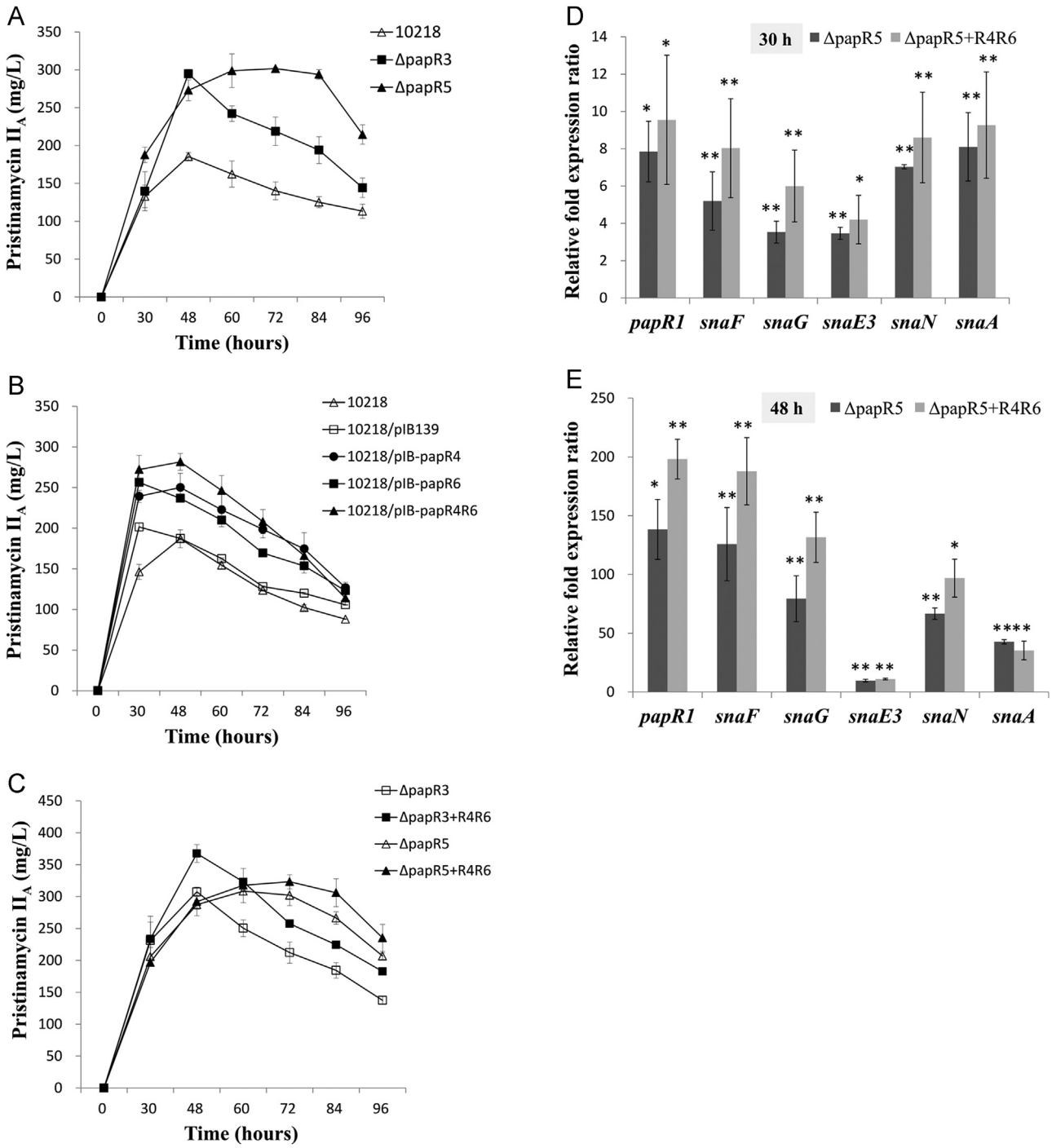


Fig. 5. The effects of engineering the cluster-situated regulatory genes on PII$_A$ production and gene expression. (A) PII$_A$ production in Δ*papR3* and Δ*papR5* compared with the parental strain HCCB10218. (B) PII$_A$ production in the overexpression strains, including 10218/pIB-*papR4*, 10218/pIB-*papR6* and 10218/pIB-*papR4R6*, compared with the parental strain HCCB10218 and 10218/pIB139. (C) PII$_A$ production in Δ*papR3*+*R4R6* and Δ*papR5*+*R4R6* compared with Δ*papR3* and Δ*papR5*, respectively. Fermentation samples were collected at the six indicated time points, and fermentations were performed in triplicates. (D, E) Transcriptional analysis of six selected PII biosynthetic genes. Fermentation samples for RNA isolation were collected at 30 and 48 h. The *hrdB* gene was used as an internal control. The relative transcription levels of the tested genes were normalized to the *hrdB* gene. The relative fold changes in the expression of each gene (Δ*papR5* and Δ*papR5*+*R4R6 versus* 10218) were determined using the $2^{-\Delta\Delta Ct}$ method. The error bars indicate the standard deviations from three independent biological replicates, $^{*}p < 0.05$ and $^{**}p < 0.01$.

after 72 h of fermentation, Δ*papR3* formed a mass of short mycelia compared with Δ*papR5*, which showed a normal mycelium phenotype, forming long vegetative mycelia (Fig. S3A).

To explore whether the deletion of these two repressor genes had a superimposition effect on PII biosynthesis, a double mutant Δ*papR3-R5* was constructed by in-frame deleting *papR5* in the mutant Δ*papR3*. Unfortunately, Δ*papR3-R5* produced less PII$_A$ than Δ*papR3* or Δ*papR5* (Fig. S3B), which could potentially be attributed to the impaired mycelial growth of the double mutant. Microscopic observations showed that compared with Δ*papR3* or Δ*papR5*, which formed relatively long mycelia, the double mutant Δ*papR3-R5* exhibited a poor growth phenotype, forming a mass of short mycelia after 48 h of fermentation (Fig. S3A).

3.4.2. Overexpression of the possible activator genes

papR1, *papR2* and *papR4*, which encode three SARP-type regulators, have been identified as playing positive roles in pristinamycin biosynthesis (Mast et al., 2011). A putative two-component response regulator gene *papR6* has been recently identified as a pathway-specific activator gene of PII biosynthesis (Dun et al., 2015). Furthermore, disruption of the γ-butyrolactone receptor gene *spbR* resulted in severe defects of pristinamycin biosynthesis and morphological differentiation (Folcher et al., 2001), suggesting that this gene may act as a positive regulator of pristinamycin production. Therefore, these five regulatory genes were individually cloned into the integrative plasmid pIB139, and their expression was under the control of the strong constitutive promoter *ermE**p. The resulting constructs were introduced into the parental strain HCCB10218, generating five strains, namely, 10218/pIB-*papR1*, 10218/pIB-*papR2*, 10218/pIB-*papR4*, 10218/pIB-*papR6* and 10218/pIB-*spbR*. HCCB10218 with the empty vector pIB139 (10218/pIB139) was used as a negative control. qRT-PCR analysis was carried out to examine whether the five regulatory genes were overexpressed. RNA samples were prepared from the fermentation cultures of the *S. pristinaespiralis* strains collected at 30 h. As expected, all five regulatory genes showed enhanced expression levels by more than 2-fold, with *papR2* exhibiting the highest transcriptional increase (approximately 34-fold) (Fig. S4A). We noticed that the increased expression of each regulatory gene had no influence on bacterial growth in the fermentation medium (data not shown).

The effects of overexpression of these five regulatory genes on PII_A production were assayed by HPLC analysis. The results revealed that the overexpression of *papR4* or *papR6* led to maximum increases in PII_A production by 35% and 38% over the parental strain, respectively (Fig. 5B), whereas little effect was observed after overexpressing *papR1* or *spbR* (Fig. S4B). Surprisingly, overexpression of *papR2* led to a significant decrease in PII_A production (Fig. S4B). A similar phenotype was also observed when *papR2* was overexpressed in the wild-type strain *S. pristinaespiralis* ATCC25486 (data not shown). These results suggested that a maximum threshold concentration for the positive role of PapR2 in PII biosynthesis by *S. pristinaespiralis* might exist. Such a phenomenon in which high expression of a pathway-specific activator results in decreased antibiotic biosynthesis has also been described for *aveR*, a LAL-family cluster-situated activator gene for avermectin biosynthesis in *Streptomyces avermitilis* (Kitani et al., 2009). PII_A production was further increased by co-overexpression of both *papR4* and *papR6*. The maximum PII_A production in the *papR4*/*papR6* overexpression strain (10218/pIB-*papR4R6*) reached 282 mg/L, showing a 52% improvement over the parental strain (Fig. 5B).

3.4.3. Combined manipulation of the regulatory genes

We sought to further improve PII titer by overexpressing both *papR4* and *papR6* in Δ*papR3* or Δ*papR5*. Two strains Δ*papR3*/pIB-*papR4R6* and Δ*papR5*/pIB-*papR4R6* were constructed, and PII_A production was measured by HPLC. Fermentation samples of four *S. pristinaespiralis* strains, Δ*papR3*/pIB139, Δ*papR3*/pIB-*papR4R6*, Δ*papR5*/pIB139 and Δ*papR5*/pIB-*papR4R6*, were collected at 48 and 72 h. HPLC analysis revealed that Δ*papR5*/pIB-*papR4R6* exhibited slightly improved PII_A production compared with Δ*papR5*/pIB139, whereas Δ*papR3*/pIB-*papR4R6* (maximum PII_A production, 366 mg/L) produced 24% more PII_A than Δ*papR3*/pIB139 (maximum PII_A production, 295 mg/L) (Fig. S5).

As pIB-*papR4R6* carries the same apramycin resistance *aac(3)IV* cassette and *attP* integration site as BAC-F1F15 (harboring the assembled PII gene cluster), Δ*papR3*/pIB-*papR4R6* and Δ*papR5*/pIB-*papR4R6* could not be engineered by duplication of the PII biosynthetic gene cluster. To address this issue, we constructed two strains Δ*papR3*+*R4R6* and Δ*papR5*+*R4R6* by integrating the *papR4* and *papR6* expression cassette (under the control of *ermE**p) into the genome of Δ*papR3* or Δ*papR5*, respectively (Fig. S1C). The genes *SSDG_02344/02345* that encode a toxin/antitoxin pair were chosen for the integration of the *papR4* and *papR6* expression cassette, as their inactivation had no effect on bacterial growth or pristinamycin production (data not shown). Identical to the maximum PII_A production obtained from Δ*papR3*/pIB-*papR4R6* and Δ*papR5*/pIB-*papR4R6*, Δ*papR3*+*R4R6* and Δ*papR5*+*R4R6* produced maximum PII_A levels of 368 and 323 mg/L, respectively, with 99% and 75% improvements over the parental strain (Fig. 5C and Fig. S5).

To investigate the effects of systematical engineering of the cluster-situated regulatory genes on PII production at the transcriptional level, we compared the transcription of six PII biosynthetic genes (as described above) in Δ*papR5* and Δ*papR5*+*R4R6* with that of the parental strain by qRT-PCR analysis. RNA samples were isolated from the fermentation cultures of HCCB10218, Δ*papR5* and Δ*papR5*+*R4R6*, which were collected at 30 and 48 h. The results indicated that the expression levels of six tested genes were significantly increased in Δ*papR5* and Δ*papR5*+*R4R6* in comparison to the parental strain (Fig. 5D and E), which was identical to the enhanced PII_A production. We also observed that overexpression of both *papR4* and *papR6* in Δ*papR5* (Δ*papR5*+*R4R6* compared to Δ*papR5*) led to a further increase in mRNA abundance for all tested genes (Fig. 5D and E). Altogether, these data revealed that PII production could be improved by systematical engineering of the cluster-situated regulatory genes.

3.5. A combined approach involving the addition of an extra copy of the PII biosynthetic gene cluster and systematical manipulation of the cluster-situated regulatory genes

A combination of the above two different metabolic engineering approaches was employed to construct higher PII-producing strains. Four strains, namely, Δ*papR3*/BAC-F1F15, Δ*papR5*/BAC-F1F15, Δ*papR3*+*R4R6*/BAC-F1F15 and Δ*papR5*+*R4R6*/BAC-F1F15, were constructed by integrating the assembled gene cluster (BAC-F1F15) into Δ*papR3*, Δ*papR5*, Δ*papR3*+*R4R6* and Δ*papR5*+*R4R6*, respectively. Four other strains, namely, Δ*papR3*/BAC-F15, Δ*papR5*/BAC-F15, Δ*papR3*+*R4R6*/BAC-F15 and Δ*papR5*+*R4R6*/BAC-F15, were generated as negative controls by introducing the control vector BAC-F15 into Δ*papR3*, Δ*papR5*, Δ*papR3*+*R4R6* and Δ*papR5*+*R4R6*, respectively. Analysis of pristinamycin production revealed that introduction of the assembled PII gene cluster into Δ*papR5* (Δ*papR5*/BAC-F1F15) and Δ*papR5*+*R4R6* (Δ*papR5*+*R4R6*/BAC-F1F15) led to the maximum production of PII_A at titers of 408 and 462 mg/L, which were approximately 118% and 150% higher over the parental strain HCCB10218 (185 mg/L) (Fig. 6A and B). However, little PII_A titer improvement was observed in Δ*papR3*/BAC-F1F15 compared with the control strain Δ*papR3*/BAC-F15, and surprisingly, Δ*papR3*+*R4R6*/BAC-F1F15 produced less PII_A titer than Δ*papR3*+*R4R6*/BAC-F15 (Fig. 6A and B). The growth analysis by the light microscope revealed that after 48 h of fermentation, compared with the control strain Δ*papR3*+*R4R6*/BAC-F15 that showed a normal mycelium formation, Δ*papR3*+*R4R6*/BAC-F1F15 formed sparse, short mycelia (Fig. S6), which was akin to the phenotype of the double mutant Δ*papR3-R5* (Fig. S3A). This growth difference might account for the lower PII titer of Δ*papR3*+*R4R6*/BAC-F1F15 in comparison to Δ*papR3*+*R4R6*/BAC-F15.

Similarly, transcriptional analysis of six PII biosynthetic genes in Δ*papR5*+*R4R6*/BAC-F15 and Δ*papR5*+*R4R6*/BAC-F1F15 compared with 10218/BAC-F15 was performed by qRT-PCR. RNA samples were isolated from the fermentation cultures collected at 30 and 48 h. The results showed that the mRNA abundance of all the tested genes was significantly enhanced in both Δ*papR5*+*R4R6*/BAC-F15 and Δ*papR5*+*R4R6*/BAC-F1F15 compared with those in 10218/BAC-F15

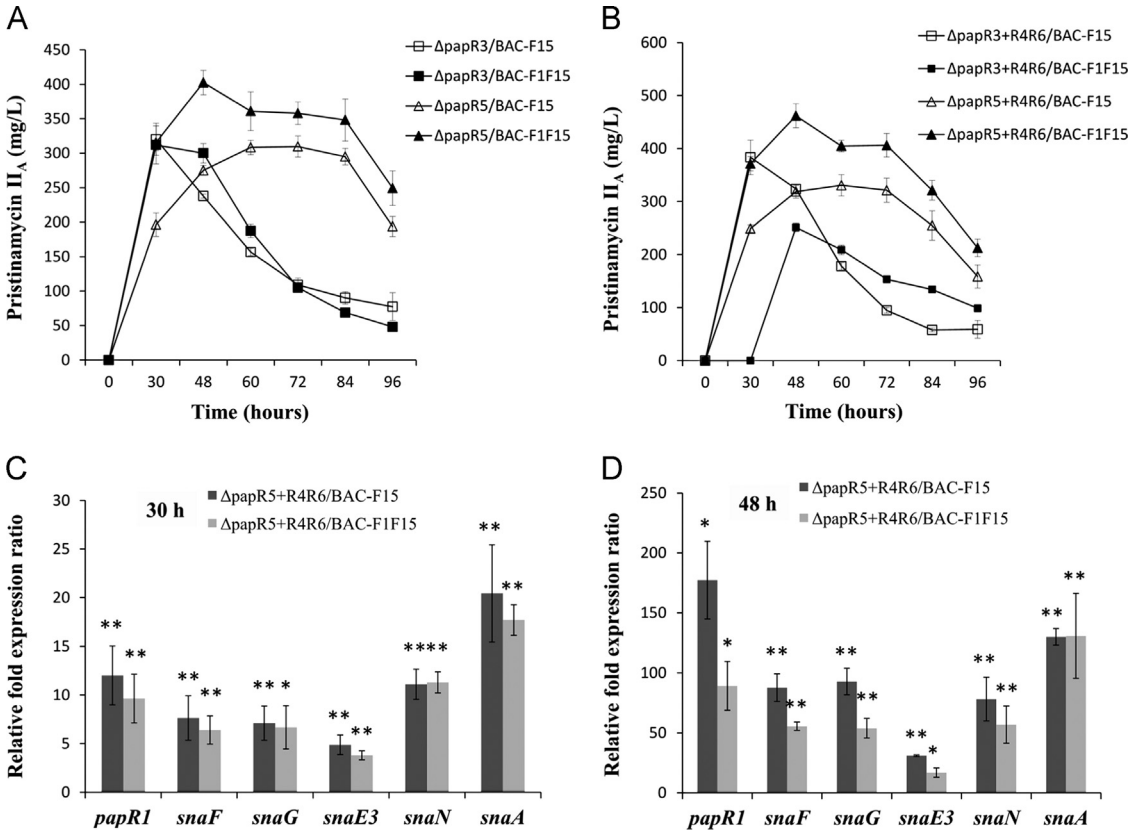


Fig. 6. The effects of introducing an assembled PII biosynthetic gene cluster combined with systematical manipulation of the cluster-situated regulatory genes on PII$_A$ production and gene expression. (A) PII$_A$ production in Δ*papR3*/BAC-F1F15 and Δ*papR5*/BAC-F1F15 strains compared with Δ*papR3*/BAC-F15 and Δ*papR5*/BAC-F15, respectively. (B) PII$_A$ production in Δ*papR3*+*R4R6*/BAC-F1F15 and Δ*papR5*+*R4R6*/BAC-F1F15 strains compared with Δ*papR3*+*R4R6*/BAC-F15 and Δ*papR5*+*R4R6*/BAC-F15, respectively. Fermentation samples were collected at six time points as indicated and fermentations were performed in triplicates. (C, D) Transcriptional analysis of six selected PII biosynthetic genes. Fermentation samples for RNA isolation were collected at 30 and 48 h. The *hrdB* gene was used as an internal control. The relative transcription levels of the tested genes were normalized to the *hrdB* gene. The relative fold changes in the expression of each gene (Δ*papR5*+*R4R6*/BAC-F15 and Δ*papR5*+*R4R6*/BAC-F1F15 *versus* 10218/BAC-F15) were determined using the $2^{-\Delta\Delta Ct}$ method. The error bars indicate the standard deviations from three independent biological replicates, $^{*}p < 0.05$ and $^{**}p < 0.01$.

(particularly at 48 h) (Fig. 6C and D). Surprisingly, although Δ*papR5*+*R4R6*/BAC-F1F15 produced 75% more PII$_A$ than Δ*papR5*+*R4R6*/BAC-F15, most of the tested genes (particularly *papR1*, *snaF*, *snaG* and *snaE3*) in Δ*papR5*+*R4R6*/BAC-F1F15 exhibited slightly reduced transcription compared with those in Δ*papR5*+*R4R6*/BAC-F15 at 48 h after fermentation (Fig. 6D). A similar reduced expression of these PII biosynthetic genes was also detected in Δ*papR5*/BAC-F1F15 compared with Δ*papR5*/BAC-F15 at 48 h after fermentation (data not shown). The phenomenon in which a higher PII production with a lower transcription of the PII biosynthetic genes might be ascribed to end-product feed-back regulation as pristinamycin could inhibit its own biosynthesis in a dose-dependent manner (Jia et al., 2006). Nevertheless, the combination of systematical manipulation of the cluster-situated regulatory genes and duplication of the PII biosynthetic gene cluster was an efficient strategy to improve PII production in *S. pristinaespiralis*.

3.6. Further improvement in PII production by adding macroreticular resin

Pristinamycin can inhibit the mycelial growth of *S. pristinaespiralis* and its own biosynthesis in a dose-dependent manner (Jia et al., 2006). Macroreticular resin can adsorb pristinamycin from the broth culture and thereby eliminate its toxic effects and feedback inhibition, providing a favorable basis for the highly efficient production of pristinamycin (Jia et al., 2006; Zhang et al., 2012). In this study, the macroreticular resin HB60 was used to optimize PII production of the engineered strain Δ*papR5*+*R4R6*/BAC-F1F15 in the Erlenmeyer flask and 5-L bioreactor. The results revealed that the parental strain HCCB10218 showed no significant improvement in PII production when the fermentation medium in the Erlenmeyer flask contained 6% HB60 resin compared with that without the resin (188 mg/L compared to 185 mg/L) (Fig. 7A). However, the engineered strain Δ*papR5*+*R4R6*/BAC-F1F15 exhibited substantially increased PII$_A$ production throughout the tested time course. The maximum PII$_A$ titer reached 1134 mg/L, a 5-fold increase over the parental strain under the same fermentation conditions (with resin) (Fig. 7A). Furthermore, the batch fermentation of HCCB10218 and Δ*papR5*+*R4R6*/BAC-F15 in the 5-L bioreactor was performed by adding HB60 resin. As shown in Fig. 7B, the engineered strain accumulated a maximum PII$_A$ level of 1158 mg/L, with a 5.26-fold improvement over the parental strain, similar to the fermentation results obtained using the Erlenmeyer flask. Thus, these results demonstrated that PII biosynthesis in the engineered strain Δ*papR5*+*R4R6*/BAC-F15 could be further elevated by adding macroreticular resin in both the flask and batch culture conditions.

4. Discussion

In this study, we reported a combinatorial metabolic engineering strategy involving the addition of an extra copy of the PII biosynthetic gene cluster in combination with systematical engineering of the cluster-situated regulatory genes to improve pristinamycin II (PII) production in *S. pristinaespiralis*. The application of these two approaches separately contributed to significantly enhanced PII$_A$ production by 45% (10218/BAC-F1F15 compared with HCCB10218)

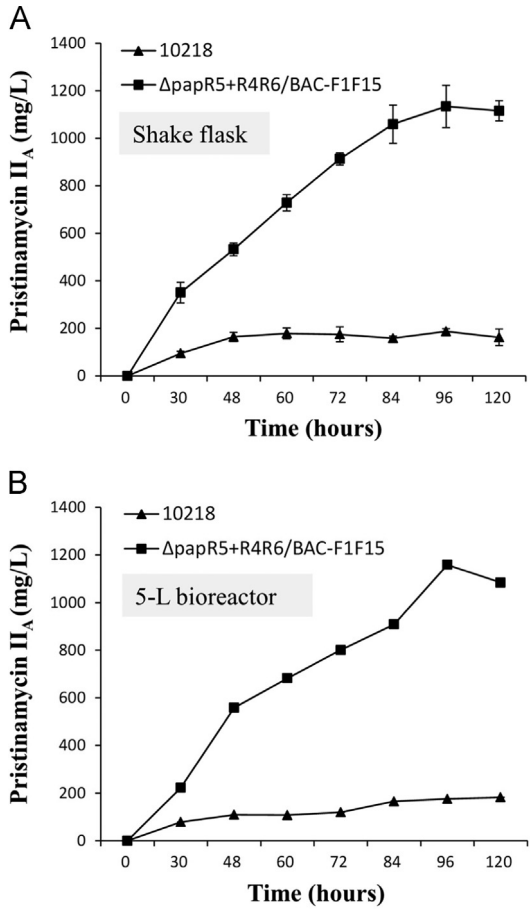


Fig. 7. The effects of adding macroreticular resin HB60 on PII_A production. PII_A production in Δ*papR5*+*R4R6*/BAC-F1F15 compared with the parental strain by adding 6% (w/v) of the resin HB60 at 24 h after fermentation in the Erlenmeyer flask (A) and 5-L bioreactor (B). Fermentation samples were collected at seven time points as indicated. Fermentations were performed in triplicates in the flask culture condition.

and 99% (Δ*papR3*+*R4R6* compared with HCCB10218). Combining these two approaches led to a higher PII-producing strain Δ*papR5*+*R4R6*/BAC-F1F15. The maximum PII_A production in the engineered strain reached 462 mg/L, which was approximately 150% higher than that of HCCB10218 (Fig. 8). The results suggested that a more relaxed regulation system of pristinamycin biosynthesis was achieved by manipulating the cluster-situated regulatory genes, which provided a favorable basis for PII improvement by introducing an extra copy of the PII biosynthetic gene cluster. The engineered strain Δ*papR5*+*R4R6*/BAC-F1F15 finally produced 1.13 g/L and 1.16 g/L PII titer in the Erlenmeyer flask and 5-L bioreactor, respectively, when the adsorbent resin was added to the fermentation culture (Fig. 8). PII production obtained in this study was comparable to the highest pristinamycin levels reported in the literature, which were 1.30 g/L and 1.01 g/L (both PI and PII) in the Erlenmeyer flask and 5-L bioreactor, respectively (Jia et al., 2008). Notably, the previously reported data were achieved by medium optimization, which provided an alternate option for the further improvement of the engineered strain described in this work.

We showed here that appropriately combining two different approaches is better than using a single approach to improve PII production in *S. pristinaespiralis*, which can be extended to other industrially used streptomycetes for strain improvement. Based on this consideration, other different metabolic engineering strategies, such as increasing the precursor supplies (Olano et al., 2008; Zabala et al., 2013) and overexpressing the genes encoding antibiotic transporters to reduce the end-product toxic effects and feedback inhibition (Jin et al., 2010; Martin et al., 2005), will be combined in the engineered strain we constructed here to further optimize PII biosynthesis. Herein, we focused only on the improvement of PII production. However, considering that the clinically used pristinamycin needs a mixture of PI and PII (normally 30:70), which act synergistically to show potent antibacterial activity at an approximately 100-fold higher level than that of either single component (Giambattisti et al., 1989), the construction of a high PI-producing strain will also be necessary in the future.

An interesting phenomenon was observed in which Δ*papR3* and Δ*papR5*, despite accumulating almost the same maximum PII titers, showed very different bacterial growth phenotypes in the fermentation medium (Fig. 5A and Fig. S3A). Compared with Δ*papR5*, which formed long vegetative mycelia before 72 h, Δ*papR3* exhibited similarly long mycelium formation, but only before 48 h (Fig. S3A). As reported, pristinamycin shows strong toxic effects on the bacterial growth of the producing strain (Contreras and Vázquez, 1977; Jia et al., 2006), and the degree of toxic effects may depend on the PI/PII ratio. We found that Δ*papR3* produced higher PI titers (101 mg/L) than Δ*papR5* (17 mg/L) (Fig. S7A). An additional bioassay using *B. subtilis* as the indicator strain demonstrated that the culture filtrate of Δ*papR3* had a much higher bactericidal activity than that of Δ*papR5* (Fig. S7B). Thus, the growth difference between Δ*papR3* and Δ*papR5* might be mainly due to the different levels of toxic effects exerted by the combination of PI and PII produced in these two strains. In addition, we observed that either deleting *papR5* in Δ*papR3* or introducing an extra PII biosynthetic gene cluster into Δ*papR3*+*R4R6* led to reduced PII production as well as poor mycelial growth after 48 h of fermentation (Fig. S3A and Fig. S6). We speculated that the simultaneous inactivation of the two TetR-family repressors or the duplication of the PII biosynthetic gene cluster based on Δ*papR3*+*R4R6* might have resulted in a precocious and higher transcription of the PII biosynthetic genes, which disturbed primary metabolism and then affected the bacterial growth at an early stage. This incoordination might finally lead to the lower PII titers in these two engineered strains, Δ*papR3-R5* and Δ*papR3*+*R4R6*/BAC-F1F15. Altogether, the cluster-situated regulators and their interactions played very important roles in fine-tuning pristinamycin biosynthesis, thereby influencing mycelium formation indirectly. An in-depth investigation into the regulation mechanism of these regulators will be an interesting topic in the future.

Finally, a modified Gibson assembly method was developed to clone the PII biosynthetic gene cluster. This method could be expanded to the assembly of large DNA fragments with high GC contents, such as secondary metabolite biosynthetic gene clusters from actinomycetes. The key step for improving this method was to add two DNA overhangs (with low AT contents) to the ends of the cloning vector instead of the high GC overhangs used in the classical Gibson assembly method, which significantly reduced the vector self-ligation ratio from 80% to 45%. Although the assembly efficiency increased from 2.5% to 20–40% using our modified method, further optimization remains required, such as appropriately extending the length of the overlaps between the DNA inserts and the vector. This modified Gibson method provides an effective strategy to clone the large high GC gene cluster, which is a useful supplement to other methods reported previously (Cobb and Zhao, 2012), such as the DNA assembler method (Shao and Zhao, 2009) and the RecET or TAR-mediated direct cloning methods (Fu et al., 2012; Yamanaka et al., 2014).

5. Conclusions

A combination of two different metabolic engineering approaches, namely the duplication of the PII biosynthetic gene cluster and systematical engineering of the cluster-situated

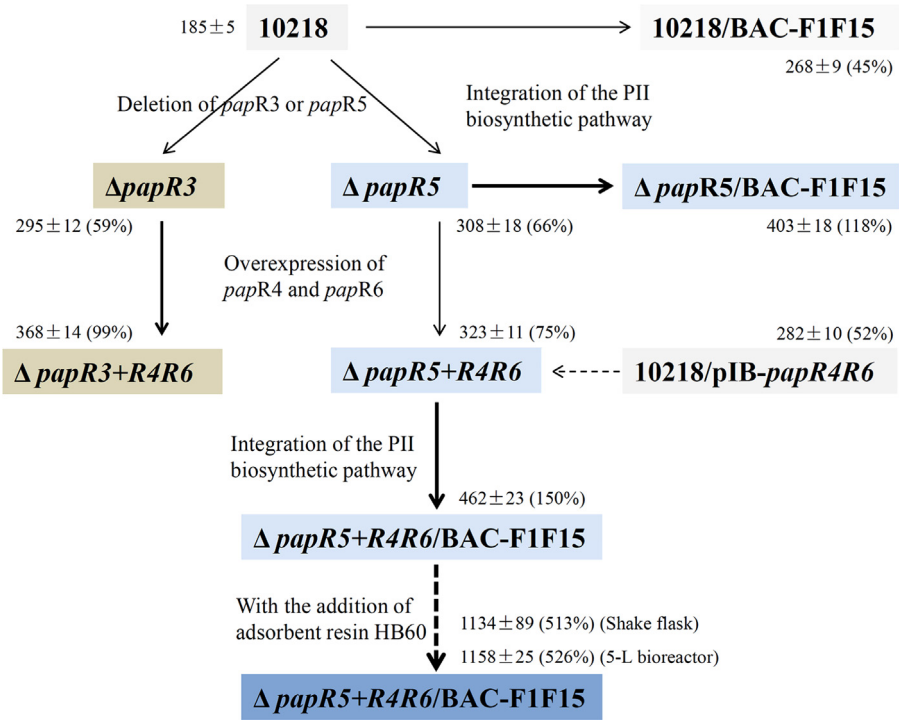


Fig. 8. The genetic lineage and PII_A production of the constructed strains in this study. The maximum PII_A titers of the constructed strains and the corresponding titer rates of increase in comparison to the parental strain are presented. Fermentations were performed in triplicates for each strain.

regulatory genes, was employed in the present study to optimize PII production, which led to a maximum PII titer improvement of approximately 1.5-fold higher compared with that of the parental strain. After adding 6% macroreticular resin, the maximum PII levels of the engineered strain Δ*papR5*+*R4R6*/BAC-F1F15 reached 1.13 and 1.16 g/L in the Erlenmeyer flask and 5-L bioreactor, respectively, with 5.13- and 5.26-fold higher improvements over the parental strain. In addition, we developed a modified Gibson assembly method that was optimized for the assembly of large DNA fragments with high GC contents, which will benefit the rapid cloning of secondary metabolite biosynthetic gene clusters from actinomycetes.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2015.02.001.

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