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Overproduction of gentamicin B in industrial strain *Micromonospora echinospora* CCTCC M 2018898 by cloning of the missing genes *genR* and *genS*

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ABSTRACT

In pharmaceutical industry, isepamicin is mainly manufactured from gentamicin B, which is produced by *Micromonospora echinospora* as a minor component of the gentamicin complex. Improvement of gentamicin B production through metabolic engineering is therefore important to satisfy the increasing demand for isepamicin. We hypothesized that gentamicin B was generated from gentamicin JI-20A via deamination of the C2' amino group. Using *kanJ* and *kanK* as the gene probes, we identified the putative deamination-related genes, *genR* and *genS*, through genome mining of the gentamicin B producing strain *M. echinospora* CCTCC M 2018898. Interestingly, *genR* and *genS* constitute a gene cassette located approximately 28.7 kb away from the gentamicin gene cluster. Gene knockout of *genR* and *genS* almost abolished the production of gentamicin B in the mutant strain, suggesting that these two genes, which are responsible for the last steps in gentamicin B biosynthesis, constitute the missing part of the known gentamicin biosynthetic pathway. Based on these finding, we successfully constructed a gentamicin B high-yielding strain (798 mg/L), in which an overexpression cassette of *genR* and *genS* was introduced. Our work fills the missing piece to solve the puzzle of gentamicin B biosynthesis and may inspire future metabolic engineering efforts to generate gentamycin B high-yielding strains that could eventually satisfy the need for industrial manufacturing of isepamicin.

1. Introduction

Aminoglycosides (AGs) constitute one of the oldest classes of clinically important antibiotics (Becker and Cooper, 2013; Jackson et al., 2013; Piepersberg et al., 2007). By acting specifically on the bacterial 30S ribosomal subunit and further interfering with protein synthesis (Carter et al., 2000; Moazed and Noller, 1987; Scheunemann et al., 2010), AGs show excellent activity against a wide variety of Gram positive and negative pathogens. This allows for broad clinical application of this class of compounds to treat microbial infections in livestock and humans (Arya, 2007; Magnet and Blanchard, 2005). Decades of AG usage in clinical practice resulted in the worldwide spread of resistant pathogens that have evolved various AG modifying enzymes (AMEs) to deactivate these antibiotics (Garneau-Tsodikova and Labby, 2016; Ramirez and Tolmasky, 2010). However, inspired by the natural AMEs-defensive structural features of some AGs, scientists have developed second-generation semisynthetic AGs that are less susceptible to the common AMEs (Kondo and Hotta, 1999;Cunha, 2006).

Isepamicin is one of the second-generation AGs, which was approved to the market in 1988 (Nagabhushan et al., 1978). It possesses a high level of stability to AMEs, and exhibits antimicrobial activity comparable to other first-line AG drugs but with lower ototoxicity and nephrotoxicity (Jones, 1995). Although several synthetic methods for isepamicin production have been developed (Moon et al., 2005; Nagabhushan et al.,

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1978), N-acylation of gentamicin B with (*S*)-3-amino-2--hydroxypropionic acid is the major strategy employed by pharmaceutical industry to produce isepamicin (Kumar et al., 2008). Gentamicin is a group of 2-deoxystreptamine (2-DOS)-containing AGs isolated from *Micromonospora*, and gentamicin B is co-produced in the gentamicin fermentation as a minor product (Testa and Tilley, 1976; Weinstein et al., 1963). Improvement of gentamicin B production in the host strain can thus satisfy the demand for decreasing the cost of isepamicin manufacturing.

Ultraviolet (UV) mutagenesis combined with serial passage has been widely used to screen gentamicin B high-yielding strains for several decades. Although the desirable strains can be generated via this method, the breeding process is expensive and laborious. With the elucidation of the biosynthetic pathway of gentamicin, combinatorial biosynthesis or pathway engineering can be attractive alternatives to improve the production of gentamicin B in an efficient way (Park et al., 2017). It has been

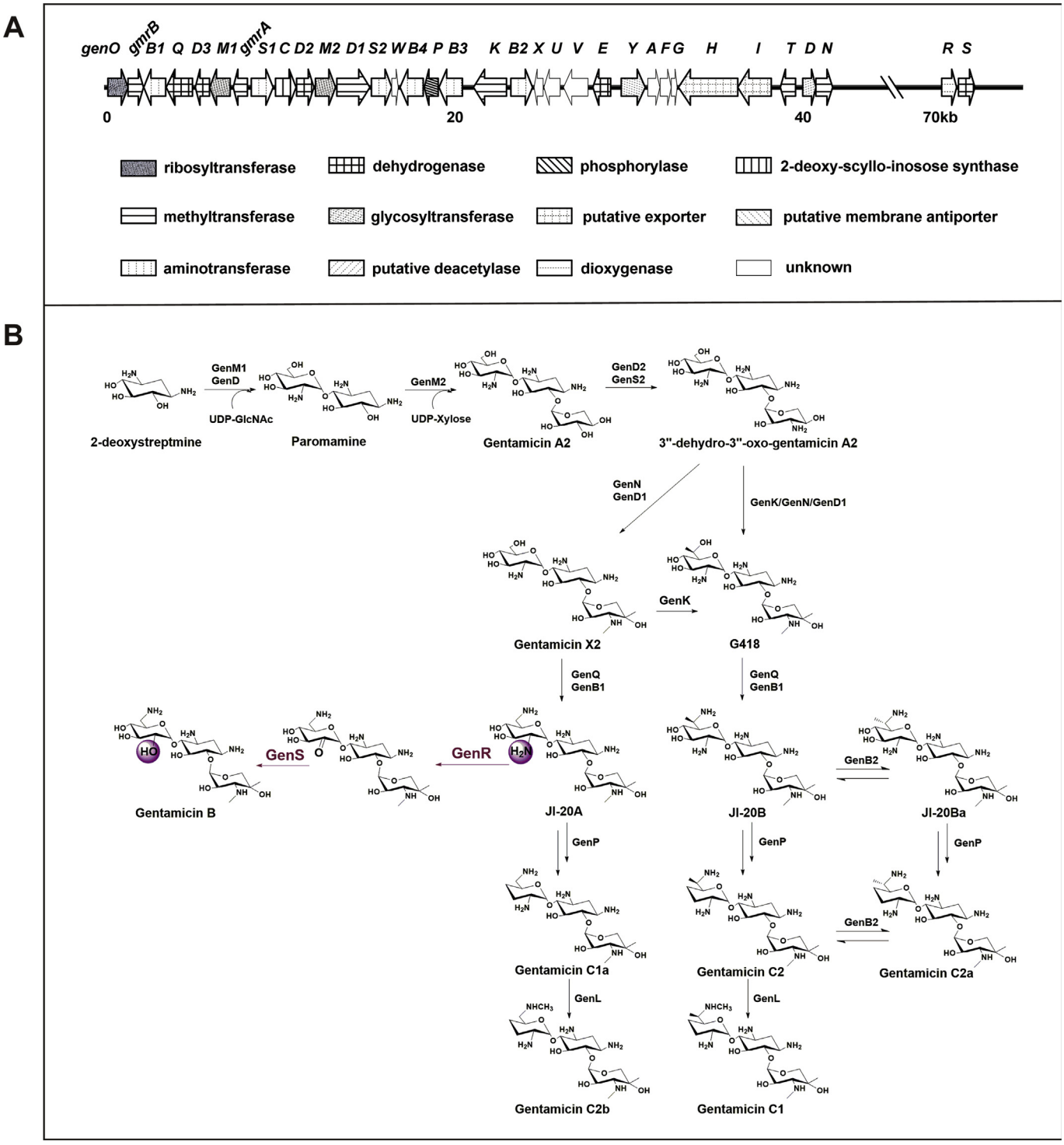

Fig. 1. Biosynthesis gene cluster of gentamicin C complex and the predicted biosynthetic pathway of gentamicin B in *M. echinospora* CCTCC M 2018898.
(A) Synthetic gene cluster of gentamicin C complex predicted based on sequencing results in *M. echinospora* CCTCC M 2018898. (B) Biosynthetic pathway of gentamicin C complex and the predicted biosynthetic pathway of gentamicin B.

demonstrated that the production of multiple gentamicin products in *Micromonospora echinospora* is the result of parallel biosynthetic routes (Fig. 1B) (Ban et al., 2019; Gu et al., 2015; Guo et al., 2014; Huang et al., 2015; Li et al., 2018; Yu et al., 2017). As the common biosynthetic precursor of AGs, 2-DOS is firstly attached with purpurosamine and garosamine at positions C4 and C6, respectively, to give gentamicin A2, which is the first pseudotrisaccharide precursor of the gentamicin C complex (C1, C2, C1a, C2a, and C2b). In the following steps, gentamicin A2 is converted to 3″-dehydro-3″-oxo-gentamicin A2 by the dehydrogenase GenD2 and the pyridoxal phosphate (PLP)-dependent aminotransferase GenS2. Then 3″-dehydro-3″-oxo-gentamicin A2 generates gentamicin X2 and 418 through a staggered methylation network of three methyltransferases GenN, GenD1 and GenK (Li et al., 2018). At this stage, gentamicin X2 and G418 can be further transformed into other gentamicin products via two parallel routes. Gentamicin X2 is aminated at C6′ to give JI-20A by the sequential actions of the dehydrogenase GenQ and the aminotransferase GenB1. The same set of enzymes also accepts gentamicin G418 as the substrate and converts it to JI-20B. A group of uncharacterized enzymes (likely including the phosphotransferase GenP (Shao et al., 2013)) then dideoxygenate gentamicin JI-20A and JI-20B to generate gentamicin C1a and C2, respectively. The latter two compounds can be further methylated at the C-6' amino group by the methyltransferase GenL to afford gentamicin C2b and C1 (Li et al., 2018).

While the mechanisms for the biosynthesis of gentamicin C components are well characterized, the enzymes responsible for gentamicin B production are yet fully elucidated. The structure of gentamicin B only differs from that of gentamicin JI-20A in C2′ substitution, implying that gentamicin JI-20A might be the precursor of gentamicin B. We thus proposed that a deamination reaction may occur on C2′ amino group of gentamicin JI-20A during its conversion to gentamicin B (Fig. 1B). A study on the biosynthesis of kanamycin A, which contains the same purpurosamine moiety as gentamicin B, provides further support to this hypothesis. Eguchi and colleagues have demonstrated that the Fe^{II}/α-Ketoglutarate-dependent dioxygenase KanJ and the NADPH-dependent ketoreductase KanK are responsible for the conversion of the C2′ amino group in kanamycin B to the C2' hydroxyl group in kanamycin A (Sucipto et al., 2012). Based on this finding, Xia and colleagues have constructed an artificial biosynthetic pathway of gentamicin B by introducing the genes *kanJ* and *kanK* into the gentamicin JI-20A-producing mutant, generating an engineered strain that produces gentamicin B with tenfold higher yield than the wild-type strain (Ni et al., 2016). This result further supports the hypothesis that enzymes similar to KanJ and KanK may be encoded on the chromosome of the gentamicin B producing strain.

In this study, we identified two genes showing high homology with *kanJ* and *kanK* in an industrial gentamicin B producing strain *M. echinospora* CCTCC M 2018898. These two genes constitute a small cassette located outside the previously characterized gentamicin gene cluster. Knockout of these two genes almost abolished the production of gentamicin B without affecting other gentamicin components in the mutant strain, suggesting that they function specifically in gentamicin B biosynthesis. We further overexpressed these two genes by placing them under the strong promoter *kasOp** and *SRL37*, respectively, leading to a 64% increase of gentamicin B yield (798 mg/L) in the engineered strain.

2. Materials and methods

2.1. Strains and culture conditions

The plasmids and strains used in this study are shown in Table 1. *E. coli* DH10B was used as a host for cloning. *E. coli* ET12567/pUZ8002 was used for intergeneric conjugation between *E. coli* and *Micromonospora*. *M. echinospora* CCTCC M 2018898 and its mutants were cultivated on a plate medium (Corn starch 3%, yeast extract 0.5%, $NaCl_2$ 0.2%, KH_2PO_3 0.01%, KNO_3 0.3%, $CaCO_3$ 0.6%, agar 2.0%, pH 7.2) at 37 °C for strain culturing and isolation. *E. coli* was cultured in LB medium at 37 °C with the appropriate antibiotic for selection. In the fermentation

Table 1
Strains and Plasmids Used in This study.

	Description	Reference
Strain		
M. echinospora CCTCC M 2018898	Wild-type(Also known as: *M. echinospora* HS-1520-016-89)	This study
DH10B	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*)	Gibco-BRL
ET12567(pUZ8002)	*dam dcm hsdS*/pUZ8002	Hong et al., 2009
ΔRS	*genR and genS* gene knockout strain, parent strain: wild-type	This study
ΔRS -pYC005	*genR and genS* complemented strain, parent strain: ΔRS	This study
CCTCC M 2018898-pYC004	*genR and genS* gene overexpression strain, parent strain: wild-type	This study
Plasmid		
pCRISPR-Cas9	Temperature sensitive plasmid, *aac(3)IV*, *tsr*, *oriT*, P*tipA*, sgRNA scaffold and *cas9*	Tong et al. (2015)
pCRISPR-Cas9-gRNA	*genR and genS* in-frame deletion construction, pCRISPR-Cas9 carries sgRNA	This study
pCRISPR-Cas9-gRNA-ΔRS	*genR and genS* in-frame deletion construction, pCRISPR-Cas9 carries sgRNA and homologous arms	This study
pSET152	*E.coli* replicon, *att, oriT,aac(3)IV*	Bierman et al., 1992
pSET152-hrdB	*E.coli–Streptomyces* integrative shuttle vector, pSET152 carries *gusA* under the control of the P*hrdB* promoter	This study
pWHU77	*int, att, tsr,* P*ermE**	Li et al. (2018)
pYC005	ΔRS complementation plasmid construction, pWHU77 carries *genR* under the control of the P*ermE** promoter and *genS* under the control of the original promoter	This study
pYC004	*genR and genS* overexpression construction, pWHU77 carries *genR* under the control of the P*kasOp** promoter and *genS* under the control of the P*SRL37* promoter	This study
pOJ260	pUC18 replicon, *oriT*, *aac(3)IV*	Bierman et al., 1992
pJTU1278	*bla*, *tsr*, *lacZ*, *oriT*, ori^{pIJ101}, and ori^{ColE1}	He et al. (2010)
p2-GUS	*E.coli–Streptomyces* integrative shuttle vector, pSET152 carries *gusA* under the control of the P*2* promoter	Liu et al. (2016)
p139-GUS	*E.coli–Streptomyces* integrative shuttle vector, pSET152 carries *gusA* under the control of the P*ermE** promoter	
pS1	*E.coli–Streptomyces* integrative shuttle vector, pSET152 carries *gusA* under the control of the P*ksaOp-rpsL-CF* promoter	
pL3	*E.coli–Streptomyces* integrative shuttle vector, pSET152 carries *gusA* under the control of the P*SPL42* promoter	
pL6	*E.coli–Streptomyces* integrative shuttle vector, pSET152 carries *gusA* under the control of the P*SPL39* promoter	
pR3	*E.coli–Streptomyces* integrative shuttle vector, pSET152 carries *gusA* under the control of the P*SRL39* promoter	
pR4	*E.coli–Streptomyces* integrative shuttle vector, pSET152 carries *gusA* under the control of the P*SRL37* promoter	
pN1	*E.coli–Streptomyces* integrative shuttle vector, pSET152 carries *gusA* under the control of the P*kasOp** promoter	
pN2	*E.coli–Streptomyces* integrative shuttle vector, pSET152 carries *gusA* under the control of the P*resLp-cf* promoter	
pN3	*E.coli–Streptomyces* integrative shuttle vector, pSET152 carries *gusA* under the control of the P*rpslp-TP* promoter	
pN4	*E.coli–Streptomyces* integrative shuttle vector, pSET152 carries *gusA* under the control of the P*gapdhp-KR* promoter	

experiment, a seed culture was cultured in seed medium (Corn starch 3%, soybean flour 1.5%, peptone 0.5%, KNO_3 0.03%, $CaCO_3$ 0.4%, pH 6.8~7.2) at 34 °C with shaking at 250 rpm for 36 h, then transferred (10% (v/v) inoculum) to the fermentation medium (Corn starch 4%, soybean flour 3%, peptone 0.5%, KNO_3 0.05%, $CaCO_3$ 0.6%, $MgSO_4$ 0.2%, $CoCl_2$ 0.008‰, pH 6.8~7.2) at 34 °C with shaking at 250 rpm for 5–7 d.

2.2. Complete genome sequencing and assembly

The genome sequencing of *M. echinospora* CCTCC M 2018898 was performed using PacBio RSII technology at Wuhan Institute of Biotechnology. One single-molecule real-time sequencing (SMRT) cell was used. The resulting sequence reads were assembled using the Hierarchical Genome Assembly Process (HGAP) method (Chin et al., 2013). An interpolated Markov model was used for whole-genome gene predictions. Protein-coding sequences (CDS) were predicted using GLIMMER 3.0, and protein functions were predicted using BLASTP homology alignment (E-values<$1e^{-5}$) (Delcher et al., 1999). Each gene was annotated according to biological functions deposited in the orthologous groups (COG) database. The synthesis gene cluster of secondary metabolites was predicted by using antiSMASH software (Weber et al., 2015).

2.3. Construction of gene knockout mutant

The plasmid pCRISPR-Cas9, a high-efficiency gene editing plasmid in *Streptomyces*, was used as the vector for gene knockout (Tong et al., 2015). In order to achieve gene-targeted cleavage and homologous recombination, it is necessary to insert a sgRNA recognition sequence and two homology arms of the target gene on this plasmid. Firstly, the sgRNAcas9.jar software (No.2015R11L070897) was used to predict the applicable 20 nt targeting sequences of sgRNA, followed by CATGC-CATGGN$_{20}$GTTTTAGAGCTAGAAATAGC (N$_{20}$: 20 nt targeting sequence) to generate sgRNA-F. As previously reported, the complete sgRNA sequence was inserted between *Nco*I and *Sna*BI sites of pCRISPR-Cas9 by using primers sgRNA-F/R to generate pCRISPR-Cas9-gRNA. Secondly, two homology arms of the gene knockout plasmid were amplified from *M. echinospora* CCTCC M 2018898, and then ligated by OE-PCR. The ligated homologous arm was assembled with pCRISPR-Cas9 by *Stu*I digestion using the Gibson one-step assembly kit (2x Hieff Clone MultiS Enzyme Premix, Yeasen, China) to generate the complete gene knockout plasmid pCRISPR-Cas9-gRNA-ΔRS. The primer sequences used for plasmid construction are shown in Table 2.

After sequencing verification, pCRISPR-Cas9-gRNA-ΔRS was introduced to *M. echinospora* CCTCC M 2018898 by conjugation from *E. coli* ET12567 (pUZ8002). The mixed bacterial solution were cultured in ABB13 medium (soytone 0.5%, soluble starch 0.5%, $CaCO_3$ 0.3%, MOPS 0.21%, $FeSO_4$ 0.012‰, thiamine-HCl 0.01‰, agar 2.0%) with addition of 30 mM $MgCl_2$ solution for 16–18 h at 30 °C. The plates were supplemented with apramycin (25 μg/mL) and trimethoprim (40 μg/mL). After 7 days of incubation at 30 °C, the exconjugants were screened using apramycin (50 μg/mL) and trimethoprim (50 μg/mL). Further, exconjugants were transferred to the plate solid medium containing thiostrepton (10 μg/mL) to induce the expression of Cas9. Subsequently, the mutants were verified by PCR (Fig. 4B) and sub-cultured for about two generations at 42 °C without antibiotics to lose the plasmid. Single colonies were simultaneously cultured on plate medium containing apramycin (25 μg/mL) and antibiotic-free to verify the loss of resistance.

2.4. Gene complementation of the ΔRS mutant

The plasmid pWHU77 (the pIB139 derivative, apramycin resistance gene is replaced by thiostrepton and ampicillin resistance gene) (Li et al., 2018) was used as a vector for gene complementation. The promoter *ermE** and the original promoter of *genS* were used to control expression of *genR* and *genS*, respectively. The *genR*-genS DNA fragment was amplified from *M. echinospora* CCTCC M 2018898 using primers 005-genR-F/005-genS-R (Table 2). The obtained DNA fragment and pWHU77 were then digested with *Nde*I and *Eco*RI, and ligated to generate pYC005. After sequence verification, pYC005 was introduced into ΔRS by conjugation. The exconjugants were screened using the plate medium containing thiostrepton (25 μg/mL) and verified by PCR (Fig. 5C).

Table 2
Oligonucleotide Primers Used in This study.

Primer	Sequence(5′ to 3′)	Restriction site
sgRNA-F	CATGCCATGGCCTTCGCGATCCAATGAGCAGTTTTAGAGCTAGAAATAGC	*Nco*I
sgRNA-R	ACGCCTACGTAAAAAAAGCACCGACTCGGTGCC	*Sna*BI
ΔRS-leftarm-F	AAGGCCGCTTTTGCGGGATCTCGTCGAAGGCACTAGAAGGGCGGGTCACGGCGACCTGC	
ΔRS-leftarm-R	CCGGGCGGGCCCGATGGGACCGCCCGGGGAACGGACGGGACTTCTCCCCACATTTCGTG	
ΔRS-rightarm-F	GCGGCTTGGCACAACCACACGAAATGTGGGGAGAAGTCCCGTCCGTTCCCCGGGCGGTC	
ΔRS-rightarm-R	CCGTCCGGGACCCGCGCGGTCGATCCCCGCATATAGGGGTCATGCGCTGGTCCCCGTCG	
ΔRS-YZ-F	TCGCCGCCGCTCTCGAAGAAG	
ΔRS-YZ-R	TCGGCTCGGGCATTCCCACGTTC	
GusA-YZ-F	GTCCTGCGGTTCGACGCGGTGAC	
GusA-YZ-R	CATGCCGGTCCACCGCTTCTGGAG	
005-genR-F	CGCCATATGATGTCATCAGGTCATCACTCGATC	*Nde*I
005-genS-R	CGAGAATTCCTACGCGTCCGTGGGCGATC	*Eco*RI
004-kasOp*-F	GCTTGGGCTGCAGGTCGACTCTAGTATGCATTCTAGAGGAACGATCGTTGGCTGTGTTC	
004-kasOp*-R	GGTGCGGATCGACGAAGATCGAGTGATGACCTGATGACATATGGCGTATCCCCTTTCAG	
004-genR-F	AGGAGAATACGACAGGTATCTGAAAGGGGATACGCCATATGTCATCAGGTCATCACTCG	
004-genR-R	TGTTGTCAAAGCAGAGACGGTTCGAATGTGAACAGATATCATTGGATCGCGAAGGCATG	
004-SRL37-F	GACCACTTGACGTCGGAGCATGCCTTCGCGATCCAATGATATCTGTTCACATTCGAACC	
004-SRL37-R	ACCACGTCGTGTCCGGCATCGACCAGTCCACGGACCACCATATGTAGATGTCTCCTTAC	
004-genS-F	GGAGAATACGACAGTCTAAGTAAGGAGACATCTACATATGGTGGTCCGTGGACTGGTCG	
004-genS-R	GAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGATCCCTACGCGTCCGTGGGCGATC	
004-426-1-F	GGCCCGCGACGGATCGCCCACGGACGCGTAGGGATCCCAACTTAATCGCCTTGCAGCAC	*Bam*HI
004-426-1-R	GCTCCCGGAGACGGTCACAGCTTGTCTG	
004-426-2-F	CAACACCCGCTGACGCGCCCTGACGGGCTTG	
004-426-2-R	ACACAGGAAACAGCTATGACATGATTACGAATTCGGATCCTGTATTTAGAAAAATAAAC	*Bam*HI
004-whu77-1-F	GAACCCCTATTTGTTTATTTTTCTAAATACAGGATCCGAATTCGTAATCATGTCATAGC	
004-whu77-1-R	GTGAAGTACATCACCGACGAGCAAGGCAAG	
004-whu77-2-F	GGCCCTGGCCAGCTAGCTAGAGTCGACC	
004-whu77-2-R	TTCGAATGTGAACACAGCCAACGATCGTTCCTCTAGAATGCATACTAGAGTCGACCTGC	
004-YZ-F	TGGCCAGGAGAATACGACAGG	
004-YZ-R	AGACGGTTCGAATGTGAACAG	

2.5. Construction of gene overexpression mutant

The genes *genR* and *genS* were inserted into pWHU77 for overexpression under the control of promoters *kasOp** and *SRL37* that were amplified from pN1 and pR4, respectively. The genes *genR* and *genS* were firstly amplified from *M. echinospora* CCTCC M 2018898. Vector fragment and yeast element were amplified from pWHU77 and pRS426, respectively. All fragments were assembled using the DNA fragment assembly method in yeast (Gibson et al., 2008). The yeast element was removed by *Bam*HI digestion to generate pYC004, which was then introduced into *M. echinospora* CCTCC M 2018898 by conjugation. The exconjugants of pYC004 were screened using plate medium containing thiostrepton (25 μg/mL) and verified by PCR (Fig. 5C).

2.6. Promoter strength determination based on GusA activity

Eleven constitutive promoters were selected based on the existing promoter library for *Streptomyces* (Liu et al., 2016). Additionally, using the method described in the previous chapter, *hrdB* was inserted upstream of the *gusA* gene of pSET152 to generate pSET152-hrdB. The twelve plasmids together with the control pSET152-1 (containing *gusA* without promoter) were introduced into *M. echinospora* CCTCC M 2018898 by conjugation. Positive mutants were streaked onto a plate containing apramycin (50 μg/mL) to isolate single colonies. A single colony was used for cultivation in TSB liquid medium containing apramycin (25 μg/mL) at 34 °C shaking at 250 rpm for 40 h. Thereafter, 3 mL of the culture solution was transferred to 100 mL TSB and cultured at 34 °C shaking at 250 rpm for 42 h. The activity of the reporter protein GusA was measured according to the previously reported detection method (Fig. 3A) (Siegl et al., 2013).

2.7. HPLC-CAD detection of gentamicin B

The fermentation broths of the wild type and mutant strains were adjusted to pH 1.8 with 6 N H_2SO_4 and shaken at low speed for 30 min. Before HPLC-CAD (Thermo Scientific) detection, the samples were centrifuged at 4000 rpm for 15 min, and the supernatant was filtered through 0.22 μm microporous membrane. HPLC-CAD analysis was performed using an Ultimate® LP-C18 (Welch, 250 × 4.6 mm) column with a mobile phase of 1.5% aqueous trifluoroacetic acid (phase A) and 95% methanol (phase B). Separation gradient: 0-12.5 min 100% A, flow rate: 0.8 mL/min; 12.5-15.5 min 50% A and 50% B, flow rate: 1 mL/min; 15.5-23 min 100% A, flow rate: 1 mL/min. Electrospray conditions: atomizer temperature 35 °C, sampling frequency 60 Hz.

3. Results and discussion

3.1. Genome sequencing of M. echinospora CCTCC M 2018898

M. echinospora CCTCC M 2018898 (also known as *M. echinospora* HS-1520-016-89) is an industrial gentamicin B producing strain (486 mg/L) generated by Hisun Pharmaceutical Co. Ltd through conventional UV

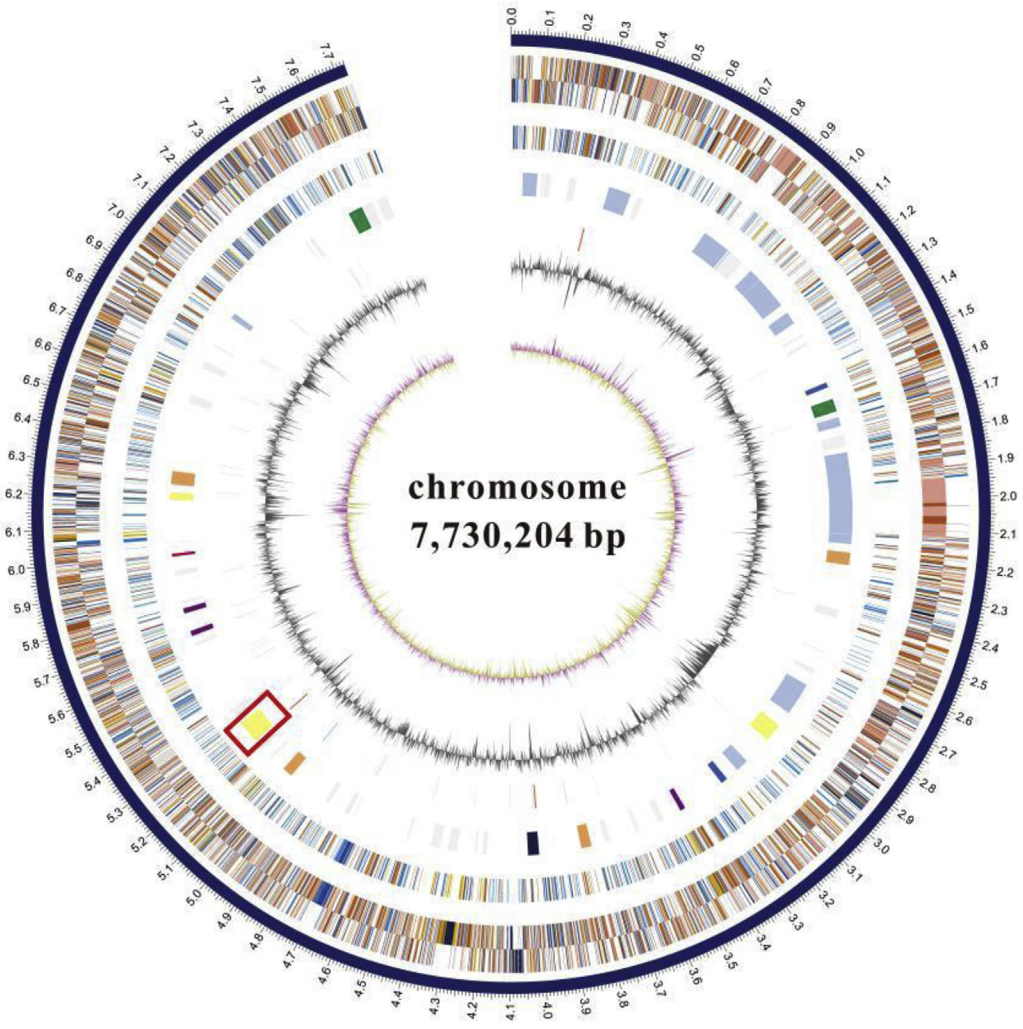

Fig. 2. Schematic representation of the *M. echinospora* CCTCC M 2018898 chromosome. From the outside in, circles 1 and 2: predicted genes (reverse and forward strands, respectively) colored according to cluster of orthologous groups (COG) function categories; circle 3: essential genes (cell division and chromosome partitioning, replication, transcription, translation, amino acid/nucleotide transport and metabolism, color coding as for circles 1 and 2); circle 4: biosynthetic gene clusters(Red frame: predicted gentamicin synthetic gene cluster); circle 5: tRNA and rRNA (blue and red, respectively); circle 6: GC content; circle 7: GC skew ([G-C/G + C], khaki indicates values > 0, purple values < 0).

mutagenesis breeding. To explore the biosynthetic gene cluster of gentamicin, we firstly determined the genome of *M. echinospora* CCTCC M 2018898 by PacBio single-molecule real-time sequencing (Koren et al., 2013). Assembly of the 920.57 Mb PacBio data produced one contig of 7, 730,204 bp, which is comprised of 6967 predicted CDSs with an average G + C content of 72.48% (Fig. 2). In silico analysis of the draft genome of *M. echinospora* CCTCC M 2018898 using antiSMASH (Weber et al., 2015) allowed the identification of a gene cluster containing 32 open reading frames (Fig. 1A), which show the same gene organization pattern and share on average 96% sequence identity with genes in the previously characterized gentamicin gene cluster of *M. echinospora* ATCC15835 (GenBank accession number: KY971520) (Guo et al., 2014).

3.2. Establishment of genetic manipulation system in M. echinospora CCTCC M 2018898

To establish the genetic manipulation method for *M. echinospora* CCTCC M 2018898, we first tried two commonly used shuttle vectors pOJ260 and pJTU1278 (He et al., 2010) to perform the conjugation experiments. The vector pOJ260 was finally successfully transferred into *M. echinospora* CCTCC M 2018898 but pJTU1278 was not (data not shown). However, after obtaining a single-crossover mutant strain through pOJ260, it was unable to screen out the double-crossover mutant strain until six-rounds of subculture. At the same time, only half of the double-crossover strains are the correct mutants (Fig. 4B). The above method is therefore very time consuming and labor intensive when it was applied in genetic manipulation in *M. echinospora* CCTCC M 2018898. Then, we chose to apply the approach based on the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system. In comparison to the conventional gene knockout method for actinomycetes that uses RecA mediated double-crossover events with nonreplicative or temperature sensitive plasmids containing long homology regions, CRISPR-Cas9 increases the screening frequency of unmarked mutants and shortens the time required to generate them (Tao et al., 2018). We then chose the temperature sensitive vector pGM1190 derived pCRISPR-Cas9 to perform the subsequent gene knockout experiments (Tong et al., 2015), and optimized the protocol by adjusting a series of parameters, including the volume ratio of donor cell and recipient cell (2:1, 4:1, 8:1), incubation time (14 h, 16 h, 18 h), and the concentration of thiostrepton (10 μg/mL, 25 μg/mL, 50 μg/mL) to induce Cas9 expression (Table 3).

3.3. Determination of promoter strength in M. echinospora CCTCC M 2018898

Rationally designed and fully characterized control elements are valuable tools for metabolic engineering methods such as gene expression fine-tuning (Boyle and Silver, 2012; Lynch and Gill, 2012; Xu et al., 2013). Among these control elements, the promoter is a key regulator of gene expression (Hammer et al., 2006). Replacing the original promoter

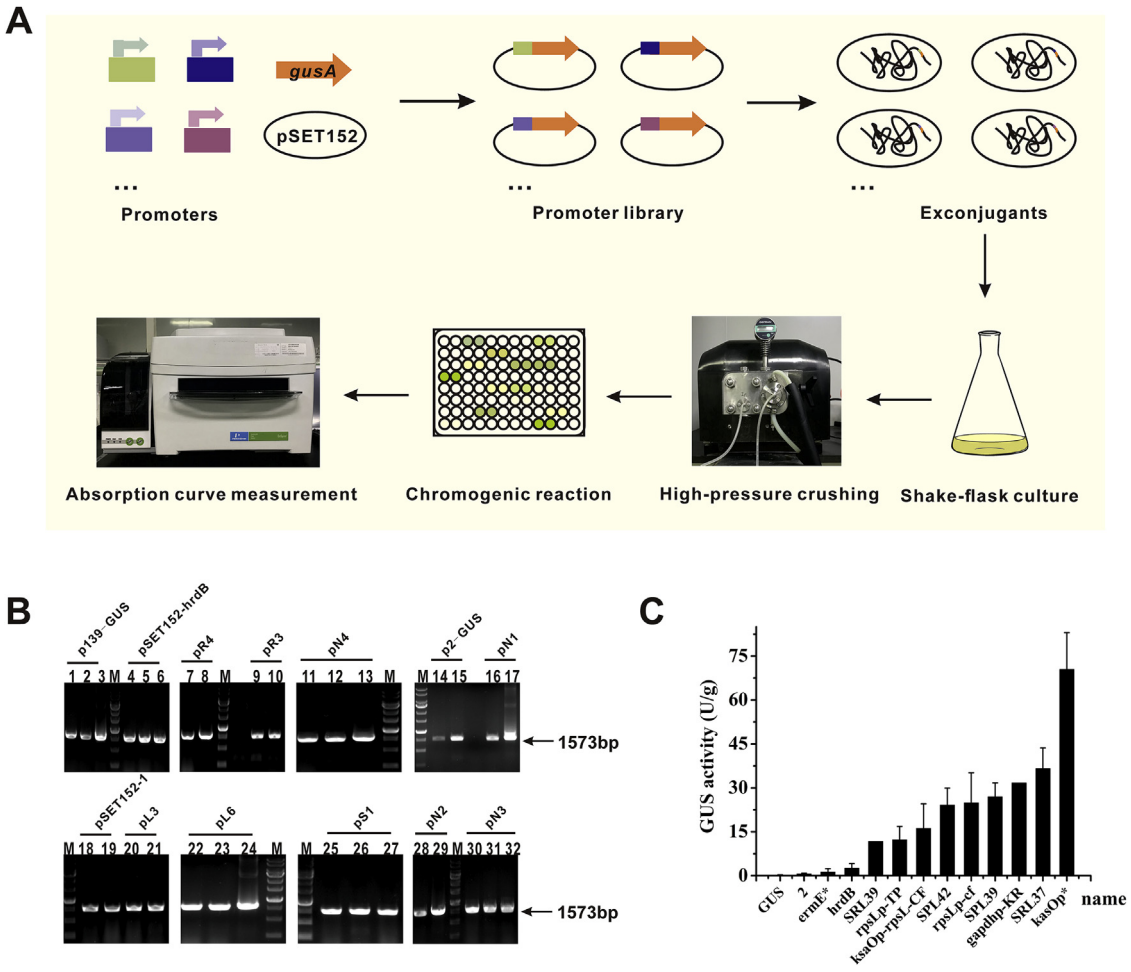

Fig. 3. Determination of promoters strength in *M. echinospora* CCTCC M 2018898.
(A) Flowchart for determination of promotersstrength based on GusA activity. (B) Confirmation of the promoter library by PCR. The PCR products were amplified using GUS-YZ-F/R. The arrow indicates the expected size of the PCR fragments in the mutants. Primer sequences are given in Table 2 (C) Strength of selected promoters in *M. echinospora* CCTCC M 2018898. All data are mean values of three independent experiments and error bars indicate the standard deviation.

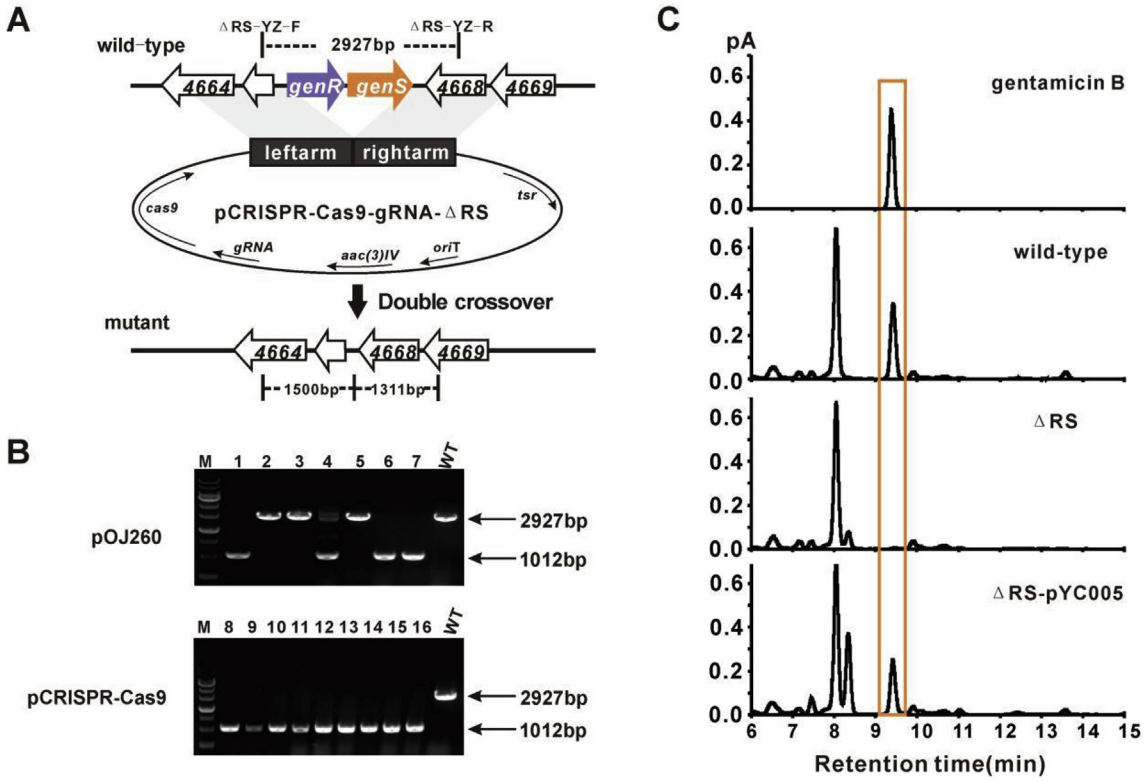

Fig. 4. Construction and HPLC-CAD analysis of *genR* and *genS* knockout strains.
(A) Schematic diagram of the in-frame deletion. (B) Confirmation of *genR and genS* knockout mutants by PCR. The PCR products were amplified using ΔRS-YZ-F/R. Among them, lane 1,6,7,8–16 were the target mutants. Lane 2,3,5 were wild-type strains. Lane 4 was the single crossover mutant. The arrows indicate the expected size of the PCR fragments in the wild-type and mutants. Primers sequences are given in Table 2 (C) HPLC-CAD analysis of gentamicin B from wild-type and mutant strains.

of the desired gene with a stronger one is an efficient way to enhance gene expression. Researchers have successfully constructed multiple promoter libraries and characterized their activities (Bai et al., 2015a; Jin et al., 2017; Siegl et al., 2013). Our previous study also identified dozens of constitutive promoters that exhibited stronger activity than *ermE** in *Streptomyces* (Liu et al., 2016). However, in previous work, there was no report on the determination of promoter strength in *Micromonospora*, and researchers could only roughly use *hrdB* and *ermE** for gene overexpression (Ni et al., 2016; Wu et al., 2017). In this study, we tested these promoters' strengths in *Micromonospora* by measuring the relative activity of the reporter gene *gusA* which was placed downstream of each candidate promoter to provide more selectable promoter elements for subsequent gene overexpression. As shown in Fig. 3C, *kasOp** and *SRL37* exhibited the highest increase (54 and 28-fold, respectively) in promoter strength when compared to *ermE**.

3.4. Identification of genes responsible for gentamicin B biosynthesis

Extensive studies on gentamicin biosynthesis have been performed based on gentamicin gene cluster of *M. echinospora* ATCC15835 and its homologs identified from other strains (Kudo and Eguchi, 2009; Kudo and Eguchi, 2016). Yet, none of the genes in these clusters were found to be relevant for the conversion of gentamicin JI-20A to gentamicin B (Kudo and Eguchi, 2016; Park et al., 2017). Sequence analysis of the region in the immediate vicinity of the newly found gentamicin gene cluster also yielded no candidate genes (data not shown). Taken together, these results suggest that the genes specifically involved in gentamicin B biosynthesis may be located away from the known gentamicin biosynthetic gene cluster. Inspired by the fact that both gentamicin B and kanamycin A contain the purpurosamine moiety, we set out to search for candidate genes responsible for the deamination reaction at the C2' amino group of gentamicin JI-20A by using KanJ and KanK (GenBank accession number: AJ628422) as probes. Two candidate genes (designated as *genR* and *genS*, GenBank accession number: MK567884 and MK567885, respectively) were found, which show 56% and 55% protein sequence identity with *kanJ* and *kanK*, respectively. These two genes link to each other and are located 28.7 kb downstream of the gentamicin gene cluster (Fig. 1A).

We then performed a gene knockout experiment to investigate the functions of *genR* and *genS* by applying the CRISPR/Cas9 system mentioned above. The pGM1190 derived gene deletion vector pCRISPR-Cas9-gRNA-ΔRS was then constructed, which was designed to delete a DNA fragment of 1915 bp covering both *genR* and *genS* (Fig. 4A). Following the optimized protocol guiding the conjugation between *E. coli* and *Micromonospora* as well as the induction of Cas9 expression (see Materials and Method), we successfully obtained 9 Δ*genR/S* mutants, whose genotypes were confirmed by PCR analysis (Fig. 4B). To our knowledge, this is the first example of CRISPR-Cas9 being successfully applied in genome editing of *Micromonospora*.

We next selected one candidate strain (designated as ΔRS), and subjected it to the fermentation experiment. HPLC analysis of the culture extract showed that the production of gentamicin B was almost abolished in ΔRS (Fig. 4C). To further confirm the *in vivo* role of GenR/S, we constructed a Δ*genR/S* complement strain (ΔRS-pYC005), in which *genR* was overexpressed under the control of the constitutive promoter *ermE** while *genS* was expressed under its native promoter (Fig. 5A). As shown in Fig. 4C/5D, the production of gentamicin B was restored in the complementation strain (78% of the original *M. echinospora* CCTCC M 2018898). These results confirmed that *genR* and *genS* are the "missing" genes responsible for the biosynthesis of gentamicin B. In fact, this kind of disconnection of essential gene(s) from the main biosynthetic gene cluster is not unprecedented. Recently, the methyltransferase catalyzing

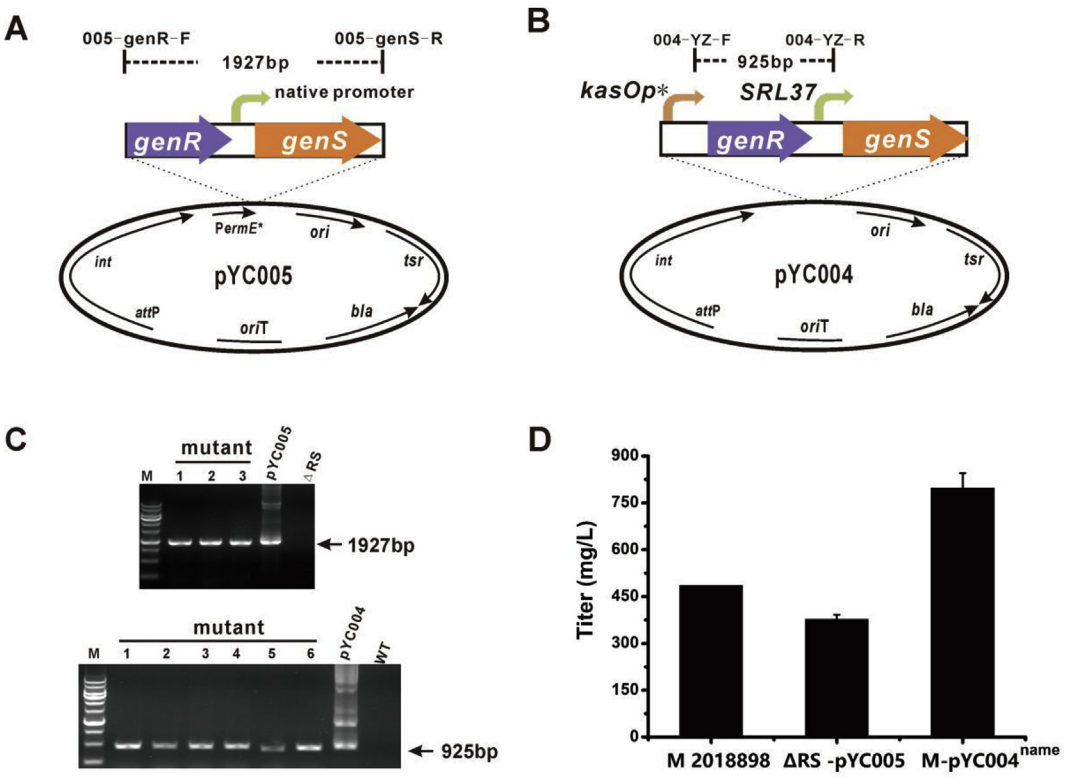

Fig. 5. Construction and product detection of *genR* and *genS* overexpression strains and complementation strains.
(A) Schematic diagram of *genR* and *genS* complementation. The *genR* is controlled by the P*ermE** promoter and *genS* is controlled by the original promoter. (B) Schematic diagram of *genR* and *genS* overexpression. The *genR* is controlled by the P*kasOp** promoter and *genS* is controlled by the P*SRL37* promoter. (C) Confirmation of mutants by PCR. The PCR products were amplified using 005-genR-F/005-genS-R and 004-YZ-F/R, respectively. The arrow indicates the expected size of the PCR fragments in mutants. Primers sequences are given in Table 2 (D) Production of gentamicin B in wild-type and mutant strains.

Table 3
Exploration of the conjugantion conditions of *E.coli* to *M. echinospora*.

Condition									
Incubation time(h)	14			16			18		
The mixing ratio(*E.coli*: *M. echinospora*)	2:1	4:1	8:1	2:1	4:1	8:1	2:1	4:1	8:1
Number of exconjugant(Cultivate to 7d)	100	240	200	100	300	260	500	500	300

6′-N-methylation of both gentamicin C2 and C1a was identified, whose encoding gene (*genL*) was found being located far from the gentamicin biosynthetic gene cluster on the chromosome of *M. echinospora* (Li et al., 2018). Taken together, the genes in charge of the conversion of all the gentamicin products have been unveiled, thus setting the stage for engineering of gentamicin biosynthetic pathway to generate a specific gentamicin component of interest.

3.5. Improvement of gentamycin B production in M. echinospora CCTCC M 2018898

Gentamicin B is produced as a minor gentamicin component. Based on the gentamicin biosynthetic pathway (Fig. 1B), the main bottleneck of gentamicin B biosynthesis is probably the low efficiency of the deamination reaction occurring on gentamicin JI-20A. Xia and colleagues have proven this hypothesis by introducing the deamination-related genes *kanJ* and *kanK* into a gentamicin JI-20A-producing mutant, thus increasing the yield of gentamicin B by ten-times in the resultant engineered strain (Ni et al., 2016). The above gene knockout experiment suggested that *genR* and *genS* might be specifically involved in the amination of gentamicin JI-20A, since very few production of gentamicin B was found in the ΔRS mutant. We thus speculated that the yield of gentamicin B would be increased if we enhance the expression of *genR/S*. Next, we constructed a *genR/S* overexpression strain (CCTCC M 2018898-pYC004), in which *genR* was expressed under the control of *kasOp** while *genS* was expressed under the control of *SRL37* (Fig. 5B). HPLC analysis of the fermentation culture extract indicated that the yield of gentamicin B in *M. echinospora* CCTCC M 2018898-pYC004 reached 798 mg/L (Fig. 5D).

Although the biosynthesis of gentamicin has been extensively studied during the past decade, genes proposed to be involved in the deamination of the C2′ amino group of gentamicin JI-20A that leads to generation of gentamicin B are yet to be fully characterized (Kudo and Eguchi, 2016). In this study, through genome mining, we found two genes in *M. echinospora* CCTCC M 2018898 that are homologous to *kanJ* and *kanK*, genes responsible for a similar deamination reaction in the biosynthesis of kanamycin. To our surprise, these two genes, *genR* and *genS*, constitute a gene cassette located separately from the known gentamicin biosynthetic gene cluster. Gene knockout of this cassette almost abolished the production of gentamicin B in the mutant strain. Recently, Yoon group reported the successful reconstitution of the diverse pathways of gentamicin B biosynthesis by verification *in vitro*, in which several limiting factors were proposed to contribute to the low production yield of gentamycin B in the wild-type *M. echinospora*, including the GenQ-B1 pair

involved in C6′-amination, the glycosyltransferase GenM2 involved in the attachment of xylose to paromamine, and the GenJ-K2 (corresponding to GenR-S in this study) pair responsible for 2′-deamination of gentamicin JI-20A (Ban et al., 2019). At the same time, our results *in vivo* confirms that *genR-S* occupy a critical position in the biosynthetic pathway of gentamicin B.

Generally, it is an effective product promotion strategy by overexpressing the rate-limiting biosynthetic genes to improve the utilization of precursors. In our previous work, we have used omics-guided techniques to identify the rate-limiting steps of spinosad biosynthesis, and successfully increased the production of spinosyn dramatically in *Streptomyces albus* J1074, which is about 1000-fold higher than the original strain (Tan et al., 2017). In this study, we engineered *M. echinospora* CCTCC M 2018898 to yield gentamicin B with titer up to 798 mg/L by overexpression of the *genR-S* gene cassette, which is approximately a 64% increase compared to *M. echinospora* CCTCC M 2018898. We further speculated that if we could engineer the metabolic flux towards gentamicin JI-20A by blocking the branch pathway, supplementing the precursor supply, increasing the conversion efficiency of the precursor based on the measured different strength promoters utilizing the efficient CRISPR/Cas9 system developed for genetic manipulation of *Micromonospora* in this study, it would lead to much higher yields of gentamicin B in the producing strain.

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