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 findings, 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 (Rondo 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.,...
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

![Diagram of Gentamicin Biosynthetic Pathway](image-url)

**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 the fi ursor of AGs, 2-DOS is 2015; Li et al., 2018; Yu et al., 2017). As the common biosynthetic pre-
hypothesis. Eguchi and colleagues have demonstrated that the FeII/C14
37
38
GenS2. Then 3
GenD2 and the pyridoxal phosphate (PLP)-dependent aminotransferase
transferases GenN, GenD1 and GenK (Li et al., 2018). At this stage,
micin products via two parallel routes. Gentamicin X2 is aminated at C6
gentamicin X2 and G418 can be further transformed into other genta-
mi
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
0.2%, KH2PO4 0.01%, KNO3 0.3%, CaCO3 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

| Strain | Description | Reference |
|--------|-------------|-----------|
| M. echinospora | Wild-type (Also known as: *M. echinospora* HS-1520-016-89) | This study |
| CCTCC M 2018898 | | This study |
| DH10B | F' mcrA Δ(mrr-hsdS8-MCR-189) | Gibco-BRL |
| ET12567/pUZ8002 | dam dcm hsdS/pUZ8002 | Hong et al., 2009 |
| ΔRS | gent and genS gene knockout strain, parent strain: wild-type | This study |
| ΔRS-pYCO05 | gent and genS complemented strain, parent strain: ΔRS | This study |
| CCTCC M 2018898-pYCO04 | | This study |
| pCRISPR-Cas9 | Temperature sensitive plasmid, aac(3)IV, tet, oriT, PApA, sgRNA scaffold and cas9 | Tong et al., 2015 |
| pCRISPR-Cas9-gRNA | | This study |
| pCRISPR-Cas9-gRNA-ΔRS | gent and genS in-frame deletion construction, pCRISPR-Cas9 carries sgRNA | This study |
| pSET152 | E. coli replicon, att, oriT, aac(3)IV | Bierman et al., 1992 |
| pSET152-hrdB | | This study |
| pWHU77 | int, att, tet, PermE | Li et al., 2018 |
| pYCO05 | ΔRS complementation plasmid construction, pWHU77 carries genS under the control of the PermE promoter and genS under the control of the original promoter | This study |
| pYCO04 | gent and genS overexpression construction, pWHU77 carries genS under the control of the PkuaOp promoter and genS under the control of the PSRL39 promoter | This study |
| pOJ260 | pUC18 replicon, oriT, aac(3)IV | Bierman et al., 1992 |
| pJTU1278 | bla, tet, lacZ, oriT, gusA, oriC101, and oriC101 | He et al., 2010 |
| p2-GUS | E. coli-S. griseus integrative shuttle vector, pSET152 carries gusA under the control of the PkuaOp promoter and gusA under the control of the PSRL39 promoter | Liu et al., 2016 |
| p39-GUS | E. coli-S. griseus integrative shuttle vector, pSET152 carries gusA under the control of the PermE promoter | This study |
| pS1 | E. coli-S. griseus integrative shuttle vector, pSET152 carries gusA under the control of the PFL342 promoter | This study |
| pL3 | E. coli-S. griseus integrative shuttle vector, pSET152 carries gusA under the control of the PSRL39 promoter | This study |
| pL6 | E. coli-S. griseus integrative shuttle vector, pSET152 carries gusA under the control of the PFL342 promoter | This study |
| pK3 | E. coli-S. griseus integrative shuttle vector, pSET152 carries gusA under the control of the PSRL39 promoter | This study |
| pR4 | E. coli-S. griseus integrative shuttle vector, pSET152 carries gusA under the control of the PFL342 promoter | This study |
| pN1 | E. coli-S. griseus integrative shuttle vector, pSET152 carries gusA under the control of the PFL342 promoter | This study |
| pN2 | E. coli-S. griseus integrative shuttle vector, pSET152 carries gusA under the control of the Prepl-c promoter | This study |
| pN3 | E. coli-S. griseus integrative shuttle vector, pSET152 carries gusA under the control of the Prepl-c promoter | This study |
| pN4 | E. coli-S. griseus integrative shuttle vector, pSET152 carries gusA under the control of the Pgapdh-KR promoter | This study |
experiment, a seed culture was cultured in seed medium (Corn starch 3%, soybean flour 1.5%, peptone 0.5%, KNO3 0.03%, CaCO3 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%, KNO3 0.05%, CaCO3 0.6%, MgSO4 0.2%, CoCl2 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-7) (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 metabolism was predicted by using antiSMASH software (Weber et al., 2015).

6.8~7.2) at 34 °C with shaking at 250 rpm for 7 days. 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.

2.3. 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 sgRNAs were designed and selected. The sgRNA sequences are designed and selected. The sgRNAs were amplified by PCR with primers sgRNA-F/R to generate pCRISPR-Cas9-gRNA. Secondly, 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-7) (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).

2.4. Gene complementation of the ΔRS mutant

The plasmid pWHU77 (the pB139 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 Ndel and EcoRI, and ligated to generate pYCY005. After sequence verification, pYCY005 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).

2.4. Gene complementation of the ΔRS mutant

| Primer | Sequence(5' to 3') | Restriction site |
|--------|-------------------|-----------------|
| sgRNA-F | CATTGCATCCGGGTTACGTTTGCAGTTTGGAATTGCAATGAATGAGTAGTATTTTTTGAGCTGAATATGC | Ncol |
| sgRNA-R | AAAGGCCAATCTCAAGGAACGCACTGCGGCAC | SnaI |
| ΔRS-leftarm-F | AGGACGCTCTTTCGGGAGATCTGCGGAACTAGGAGGTTTGCAAGGCGCCGATTCTGG | NdeI |
| ΔRS-leftarm-R | AGGCGGCTGGAACAGGGCTACAAGGAGGGAGCCGTTTGCGGAGGAA 
| ΔRS-rightarm-F | GCCGCGGCTGGAACAGGGCTACAAGGAGGGAGCCGTTTGCGGAGGAA |
| ΔRS-rightarm-R | GCCGCGGCTGGAACAGGGCTACAAGGAGGGAGCCGTTTGCGGAGGAA |
| ΔRS-YZ-F | TGGTGCGGAGGGACCTCCGTTCTC |
| ΔRS-YZ-R | TGGTGCGGAGGGACCTCCGTTCTC |
| GusA-YZ-F | GTCTGCGGAGGGACCTCCGTTCTC |
| GusA-YZ-R | GTCTGCGGAGGGACCTCCGTTCTC |
| 005-genS-R | CCGGATACCCAGCTACGTTCTCAGTACCATTCAATGAGGCATTGAGGACGGATGACCACTGGG |
| 005-genR-F | CAGGACGCTCTAGGCCGCGGCTATC |
| 004-kasOp-F | GCTGCGGAGGGACCTCCGTTCTCAGTACCATTCAATGAGGCATTGAGGACGGATGACCACTGGG |
| 004-kasOp-R | CAGGACGCTCTAGGCCGCGGCTATC |
| 004-genS-R | TGGTGCGGAGGGACCTCCGTTCTCAGTACCATTCAATGAGGCATTGAGGACGGATGACCACTGGG |
| 004-genR-F | TGGTGCGGAGGGACCTCCGTTCTCAGTACCATTCAATGAGGCATTGAGGACGGATGACCACTGGG |
| 004-SL17-F | GCACCCTGGCGGAGGGCTATC |
| 004-SL17-R | GCACCCTGGCGGAGGGCTATC |
| 004-gnsF | GAGGAATTACGCAGCTGTTCAAGAGACTACCATATGGTCTGAGGACTACCATATGAGGACGGATGACCACTGGG |
| 004-gnsS-F | GAGGAATTACGCAGCTGTTCAAGAGACTACCATATGGTCTGAGGACTACCATATGAGGACGGATGACCACTGGG |
| 004-426-1-F | GCGCGGCGGAGGGACCTCCGTTCTCAGTACCATTCAATGAGGCATTGAGGACGGATGACCACTGGG |
| 004-426-1-R | GTCTGCGGAGGGACCTCCGTTCTCAGTACCATTCAATGAGGCATTGAGGACGGATGACCACTGGG |
| 004-426-2-F | TTGGTGCGGAGGGACCTCCGTTCTCAGTACCATTCAATGAGGCATTGAGGACGGATGACCACTGGG |
| 004-426-2-R | TTGGTGCGGAGGGACCTCCGTTCTCAGTACCATTCAATGAGGCATTGAGGACGGATGACCACTGGG |
| 004-whi77-1-F | CCGGCTGGAACAGGGCTACAAGGAGGGAGCCGTTTGCGGAGGAA |
| 004-whi77-1-R | TTGGTGCGGAGGGACCTCCGTTCTCAGTACCATTCAATGAGGCATTGAGGACGGATGACCACTGGG |
| 004-whi77-2-F | CCGGCTGGAACAGGGCTACAAGGAGGGAGCCGTTTGCGGAGGAA |
| 004-whi77-2-R | TTGGTGCGGAGGGACCTCCGTTCTCAGTACCATTCAATGAGGCATTGAGGACGGATGACCACTGGG |
| 004-YZ-F | TGGTGCGGAGGGACCTCCGTTCTCAGTACCATTCAATGAGGCATTGAGGACGGATGACCACTGGG |
| 004-YZ-R | TGGTGCGGAGGGACCTCCGTTCTCAGTACCATTCAATGAGGCATTGAGGACGGATGACCACTGGG |

Table 2

Oligonucleotide Primers Used in This Study.
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 thioestrepton (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 H₂SO₄ 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: rRNA and tRNA (blue and red, respectively); circle 6: GC content; circle 7: GC skew ([G-C]/[G+C], khaki indicates values > 0, purple values < 0).](image-url)
nutagenesis breeding. To explore the biosynthetic gene cluster of gentamicin, we firstly determined the genome of \textit{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 \textit{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 \textit{M. echinospora} ATCC15835 (GenBank accession number: KY971520) (Guo et al., 2014).

3.2. Establishment of genetic manipulation system in \textit{M. echinospora} CCTCC M 2018898

To establish the genetic manipulation method for \textit{M. echinospora} CCTCC M 2018898, we firstly 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 \textit{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 \textit{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 \(\mu\)g/mL, 25 \(\mu\)g/mL, 50 \(\mu\)g/mL) to induce Cas9 expression (Table 3).

3.3. Determination of promoter strength in \textit{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 \textit{M. echinospora} CCTCC M 2018898.
(A) Flowchart for determination of promoters strength 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 \textit{M. echinospora} CCTCC M 2018898. All data are mean values of three independent experiments and error bars indicate the standard deviation.
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 \textit{ermE} in \textit{Streptomyces} (Liu et al., 2016). However, in previous work, there was no report on the determination of promoter strength in \textit{Micromonospora}, and researchers could only roughly use \textit{hrdB} and \textit{ermE} for gene overexpression (Ni et al., 2016; Wu et al., 2017). In this study, we tested these candidate promoters to provide more selectable promoter elements for subsequent gene overexpression.

### 3.4 Identification of genes responsible for gentamicin B biosynthesis

Extensive studies on gentamicin biosynthesis have been performed based on gentamicin gene cluster of \textit{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 \textit{genR} and \textit{genS}, GenBank accession number: MK567884 and MK567885, respectively) were found, which show 56% and 55% protein sequence identity with \textit{kanJ} and \textit{kanK}, respectively. These two genes link 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 \textit{genR} and \textit{genS} by applying the CRISPR/Cas9 system mentioned above. The pGM1190 derived gene deletion vector pCRISPR-Cas9-gRNA-\Delta RS was then constructed, which was designed to delete a DNA fragment of 1915 bp covering both \textit{genR} and \textit{genS} (Fig. 4A). Following the optimized protocol guiding the conjugation between \textit{E. coli} and \textit{Micromonospora} as well as the induction of Cas9 expression (see Materials and Method), we successfully obtained 9 \textit{agenR}/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 \textit{Micromonospora}.

We next selected one candidate strain (designated as \Delta RS), and subjected it to the fermentation experiment. HPLC analysis of the culture extract showed that the production of gentamicin B was almost abolished in \Delta RS (Fig. 4C). To further confirm the \textit{in vivo} role of \textit{GenR}/S, we constructed a \Delta genR/S complement strain (\Delta RS-pYC005), in which \textit{genR} was overexpressed under the control of the constitutive promoter \textit{ermE} while \textit{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 \textit{M. echinospora} CCTCC M 2018898). These results confirmed that \textit{genR} and \textit{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

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**Fig. 4.** Construction and HPLC-CAD analysis of \textit{genR} and \textit{genS} knockout strains. (A) Schematic diagram of the in-frame deletion. (B) Confirmation of \textit{genR} and \textit{genS} knockout mutants by PCR. The PCR products were amplified using \Delta RS-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.
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) partner responsible for 2'-deamination of gentamicin J1-20A (Ban et al., 2019). At the same time, our results in vivo confirm 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 spinosad 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 titers up to 796 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 J1-20A 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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