Characterization and utilization of methyltransferase for apramycin production in Streptoalloteichus tenebrarius

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Abstract: A structurally unique aminoglycoside produced in Streptoalloteichus tenebrarius, Apramycin, is used in veterinary medicine or the treatment of Salmonella, Escherichia coli, and Pasteurella multocida infections. Although apramycin was discovered nearly 50 years ago, many biosynthetic steps of apramycin remain unknown. In this study, we identified a HemK family methyltransferase, AprI, to be the 7'-N-methyltransferase in apramycin biosynthetic pathway. Biochemical experiments showed that AprI converted demethyl-aprosamine to aprosamine. Through gene disruption of aprI, we identified a new aminoglycoside antibiotic demethyl-apramycin as the main product in aprI disruption strain. The demethyl-apramycin is an impurity in apramycin product. In addition to demethyl-apramycin, carbamyltobramycin is another major impurity. However, unlike demethyl-apramycin, tobramycin is biosynthesized by an independent biosynthetic pathway in S. tenebrarius. The titer and rate of apramycin were improved by overexpression of the aprI and disruption of the tobM2, which is a crucial gene for tobramycin biosynthesis. The titer of apramycin increased from 2227 ± 320 mg/L to 2331 ± 210 mg/L, while the titer of product impurity demethyl-apramycin decreased from 196 ± 36 mg/L to 51 ± 9 mg/L. Moreover, the carbamyltobramycin titer of the wild-type strain was 607 ± 111 mg/L and that of the engineering strain was null. The rate of apramycin increased from 68% to 87% and that of demethyl-apramycin decreased from 1.17% to 0.34%.

Keywords: Streptoalloteichus tenebrarius, Apramycin biosynthesis pathway, aprI gene, Methyltransferase

Introduction

Apramycin, produced by Streptoalloteichus tenebrarius, is an aminoglycoside antibiotic that has a unique structure (Tamura et al., 2008). Frequently used as a veterinary antibiotic drug, apramycin has high antimicrobial activity on important gram-negative pathogens and is mainly used for the treatment of Salmonella, Escherichia coli, and Pasteurella multocida infections in poultry, swine, or bovine (Zhang et al., 2009; Ziv et al., 2010). Meanwhile, apramycin is a potent antibacterial with low ototoxicity, which distinguishes it from all human-used aminoglycosides. These properties have attracted many research teams’ attentions (Becker et al., 2020; Matt et al., 2012). Apramycin, carbamyltobramycin, and carbamylkanamycin B are isolated from S. tenebrarius (Koch et al., 1973). They all belong to 2-deoxystreptamine (2-DOS) aminoglycosides. Apramycin is characterized by 2-DOS, which is linked to an unusual bicyclic 3'-deoxyoctose moiety. Carbamyltobramycin and carbamylkanamycin B are the 4, 6-disubstituted 2-DOS aminoglycoside antibiotic like gentamicin. Moreover, both carbamyltobramycin and carbamylkanamycin B (3-deoxy-carbamyltobramycin) share the same biosynthetic gene cluster (GenBank accession number: AJ810851) (Ni et al., 2011). Apramycin has an independent biosynthetic gene cluster (GenBank accession number: AJ629123) (Fig. 1A). Bioinformatical analysis shows the genes for the common intermediates 2-DOS and paromamine can be easily identified both in apramycin and tobramycin biosynthetic gene clusters (Kudo, 2020). Lidavimidine is the 3'-deoxynogenated product of paromamine under the influence of AprD3 and AprD4 (Kim et al., 2016; Kudo et al., 2016; Liu et al., 2018; Lv et al., 2016). In addition, lidavimidine is oxidized by AprQ to form 6'-oxo-lidavimidine, which may be the intermediate of bicyclic 3'-deoxyoctose moiety (Wang et al., 2021). It is also confirmed that AprD3 and AprD4 are responsible for carbamyltobramycin 3'-deoxyxygenation process (Fig. 1B) (Park et al., 2011). However, the biosynthetic pathway of the unique structure of apramycin is still obscure. Here, our research shows that a 7'-N-methyltransferase, AprI, and its substrate, demethyl-aprosamine, play an important role in the conversion process.

The accumulation of intermediate metabolites is observed in apramycin fermentation products as it is in other antibiotics. According to the VICH LG 10 guideline, if the concentration of veterinary drug impurities is more than 0.1%, it should be reported and identified. The commercial apramycin examined in this study contains 0.9–1.28% of demethyl-apramycin. Therefore, demethyl-apramycin is one of the main impurities that need to be controlled. Based on the study of the biosynthetic pathways of apramycin and tobramycin, we metabolically engineered S. tenebrarius to optimize apramycin production by blocking tobramycin biosynthesis and reducing impurity production.

Materials and Methods

Strains and Growth Conditions

The plasmids and strains of this study are respectively shown in Tables S1 and S2. E. coli Top10 was used as cloning host, E. coli ET12567/pUZ8002 for intergeneric conjunction between E. coli and Streptomyces, and E. coli BL21 (DE3) was used for protein expression experiments. Wild-type S. tenebrarius and its mutants were grown on agar medium (20 g/L soluble starch, 1 g/L beef extract, 1 g/L KNO3, 0.5 g/L K2HPO4, 3H2O, 0.5 g/L NaCl, 0.5 g/L MgSO4·7H2O, 0.01 g/L FeSO4·7H2O, 15 g/L agar, pH 7.2) for sporulation and liquid CP (20 g/L glucose, 2 g/L peptone, 4 g/L yeast extract, 0.5 g/L FeSO4·7H2O, 15 g/L agar, pH 7.2) for culture and extraction of the plasmid.
**Construction of the \textit{tobM2}-Disruption Plasmids**

Based on the aforementioned sequence of the tobramycin biosynthetic gene cluster above, two DNA fragments flanking \textit{tobM2} were amplified from the genomic DNA of \textit{S. tenebrarius} (Du et al., 2004) by using M2U-P1/P2 and M2D-P3/P4. The polymerase chain reaction (PCR) products were fused and cloned into pMD18-T (Takara) and verified by restriction endonuclease digestion and sequencing. The fragment was cloned into pSPU310 to obtain the gene disruption plasmid pAP600.

**Construction of the \textit{aprI}-Disruption Plasmids**

The sequence of the apramycin biosynthetic gene cluster has been deposited in the National Center for Biotechnology...
Information under the accession number GenBank AJ629123. For deletion of the apramycin biosynthesis gene aprI, two DNA fragments flanking aprI were amplified from the genomic DNA of S. tenebrarius by using IU-P1/P2 and ID-P3/P4. The PCR products were cloned into pJL2925 to obtain plasmid pAP601, which was verified by restriction endonuclease digestion and sequencing. Then, the homogenous arm of aprI was cloned into pSPU310 to obtain the gene disruption plasmid pAP602.

**Targeted Gene Deletion**
To create double-cross deletion mutants ΔtohM2 and ΔaprI based on wild-type strain S. tenebrarius, the corresponding plasmids pAP600 and pAP602 were introduced into S. tenebrarius through conjugation, respectively, and mutants screening were carried out using the same method described before (Ni et al., 2011). The desired deletion mutants were identified by PCR using the checking primers.

**Complementation of ΔaprI Mutant Strain**
For complementation of the aprI gene mutant, pAP603 was constructed as the intermediate vector by inserting aprI into pHJK241 (Gao et al., 2017) between the Ncol and HindIII sites. Then pAP605 was constructed by inserting aprI into the vector pEAP1 (Gu et al., 2015) under the control of the constitutive promoter PhdB. The plasmid pAP605 was then introduced into ΔaprI through conjugation. Complemented exconjugants were verified on agar medium containing erythromycin (100 μg/mL) and confirmed by PCR.

**Construction of the aprI Overexpressing Strain Based on Homologous Recombination**
The fragment of PhdB, aprI, and T0 was digested from pAP603 by BgIII, and then was added to plasmid pAP600 to obtain the recombinant plasmid pAP607. Plasmid pAP607 was introduced into S. tenebrarius through conjugation using the method described before. Targeted strain named S. tenebrarius IB was confirmed by PCR.

**Extraction and Analysis of Apramycin**
Oxalic acid was added to the fermentation broths in order to precipitate calcium and magnesium ions. The pH was adjusted to 2.0 with H2SO4 and saturated for 1 hr. The acidified broth was centrifuged at 7000 r/min for 10 min, and the pH of the supernatant was readjusted to 9.0 with NH4OH. This pretreated supernatant was then centrifuged at 7000 r/min for 10 min. The supernatant was readjusted to pH 5.5 with H2SO4, applied onto a strongly acidic resin 001 × 7 (Anhui Sanxing Resin Technology), and then the bound substances were eluted with 2 mol/L NH4OH. The eluate was used for bioassays and thin-layer chromatography analysis (TLC). Propanoi–methanol–25% ammonium hydroxide (20:25:23) was used for the solvent system of TLC. The bioassay was performed with Bacillus subtilis via agar diffusion. Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed using an LC-10AT system with an evaporative light scattering detector (ELSD). Reverse C18 column (4.6×250 mm, 5 μm) was utilized with an evaporation temperature of 45°C, a nitrogen pressure of 3.5 bar, and 0.2 mol/L trifluoroacetic acid as the running water with flow 0.4 mL/min. Fermented products were quantified using HPLC-ELSD. External standard method was used for the quantification process. Apramycin and carbamyltobramycin were quantified using commercial standards (Qilu-pharma and Aladdin). The reference substance of demethyl-apramycin was purified from aprI disruption strain by ourselves. Each strain in this study was fermented three times to ensure that the experimental data were statistically significant. The yield of each strain represents the average of the concentration of the product in the three fermentation broths and was calibrated by the standard deviation (SD). The rates were obtained from the HPLC analysis of fermented production of each strain. 1H and 13C nuclear magnetic resonance (NMR) data were recorded on a Bruker AV600 at a frequency of 600 MHz using D2O as the solvent. High-resolution electro spray ionization-mass spectrometry was performed by Department of Experiment Center at Shenyang Pharmaceutical University.

**Expression and Purification of AprI**
The aprI gene was amplified from the genomic DNA of S. tenebrarius genomic DNA by PCR with pfu polymerase (BBI) and the oligonucleotides (Takara). The PCR product was purified by gel extraction and inserted into vector pET-28a (+) between the Ndel and Eori sites to create pAP606. The resulting construct was verified by DNA sequencing and then was used to transform E. coli BL21(DE3) cells using the heat-shock method. BL21(DE3)/pAP606 cells were grown in 3 mL LB medium containing kanamycin (50 mg/mL) at 37°C until an optical density (600 nm) of 0.6–0.8 was achieved, and then the cultures were induced by isopropyl-β-D-thiogalactoside (0.5 mM) at 16°C with shaking overnight. For purification, the cell pellets from 2 L culture were harvested by centrifugation at 7000 r/min for 15 min, resuspended in 20 mL of binding buffer (20 mmol/L tris-HCl, 0.5 mol/L NaCl, 5 mmol/L imidazole, pH 8.0), and then opened through ultrasonication for 30 min on ice. Supernatant was separated from cell debris by centrifugation at 12000 r/min for 40 min at 4°C and passed through a 1 mL immobilized metal ion affinity chromatography column (GE Healthcare) charged with nickel and previously equilibrated with binding buffer. After washing the column with 30 mL of binding buffer followed by 10 mL of wash buffer (20 mmol/L tris-HCl, 0.5 mol/L NaCl, 40 mmol/L imidazole, pH 8.0), the protein was eluted with elution buffer (20 mmol/L tris-HCl, 0.5 mol/L NaCl, 200 mmol/L imidazole, pH 8.0). Fractions involving the desired product were collected and dialyzed (10 mmol/L tris-HCl, 250 mmol/L NaCl, 10% glycerol, pH 8.0). Protein was concentrated to 13 mg/mL and stored in 10% glycerol at −20°C until used.

**Enzymatic Assay of AprI**
The in vitro enzymatic assay of AprI was performed by combining 17 mmol/L K2HPO4/KH2PO4 (pH 7.4), 2 mmol/L S-adenosylmethionine (SAM), 0.4 mM substrate, and 10 μmol/L purified recombinant AprI in a 500 μL total reaction volume. The reaction was incubated at 30°C and then stopped by the addition of 1× volume of chloroform, followed by vigorous vortexing to denature the proteins. The mixture was centrifuged at 10000 r/min for 5 min and the supernatant was subjected to HPLC-ELSD and MS analysis.

**RNA Isolation and the Semi-Quantitative RT-PCR Analysis**
S. tenebrarius and ΔtohM2 were cultured on a solid fermentation medium for 48 hrs. Then the mycelia was harvested and the total RNA were isolated using the Ultrapure RNA Kit (DNase I) (Cwbio). cDNA was reverse transcribed using the PrimeScript™ RT Reagent Kit (TaKaRa). The semi-quantitative RT-PCR analysis was performed. The 16S rRNA as an internal reference was amplified using the primers RT-16SrRNA-1 and RT-16SrRNA-2. Five apramycin biosynthesis genes and tohM2 were tested.
Result and Discussion
Identify Function of aprl by Gene Deletion and Complementation

Bioinformatical analysis of the apramycin biosynthetic gene cluster showed that Aprl possessed homology with known methyltransferase, sharing 35% sequence identity with the N-methyltransferase CaIM of Streptomyces chartreusis (Wu et al., 2013) and a lower degree of sequence identity with the 3’-N-methyltransferase GenN of Microonospora echinospora (29%) (Fig. S1) (Ni et al., 2016). All of them have the conserved CxGxG motif and belong to the HemK superfamily, which catalyzes methyl group transfer of a variety of substrates. Thus, we deduce that Aprl is the 7’-N-methyltransferase in apramycin biosynthesis. To clarify this hypothesis, our study carried out both genetic and biochemical analysis of Aprl.

To clarify the function of aprl, aprl gene was disrupted in the wild-type strain of S. tenebrarius. PCR amplification with wild-type strain generated a 4.8 kb fragment, while the aprl mutant strain (∆aprl) generated a 4.0 kb fragment because of a 789 bp deletion (Fig. 2A and B). The 4.0 kb fragment amplified from ∆aprl was further confirmed by DNA sequencing (data not shown). The results verified that aprl was successfully disrupted in ∆aprl. We cultured the ∆aprl to analyze its fermented products. The wild-type strain of S. tenebrarius was used as control under the same fermentation conditions. TLC coupled with an antimicrobial activity assay showed that the Aprl produced a new bioactive product 2 instead of apramycin (Fig. 2C). HPLC analysis confirmed that ∆aprl accumulated a compound 2, which was also discovered in wild-type strain and apramycin standard (Fig. 2D). Through high-resolution mass spectrometry analysis, the component 2 produced by ∆aprl was considered to be demethyl-apramycin (Fig. S2). The demethyl product was also analyzed by MS/MS (Fig. S2). The protonated fragments were similar to those of the reported mass spectra of apramycin. However, the glycoside bond of this compound cleavage formed the fragment a + b (m/z 365) instead of the ion at m/z 379 (Park et al., 2010), suggesting the demethyl-apramycin might lack a methyl group at 7’-N. Compound 2 then was purified from the fermentation broth of ∆aprl. The 13C-NMR analysis definitively confirmed that compound 2 lacked a 7’-N-methyl group (Fig. S3 and Table S4). Compared to the wild-type strain, the demethyl-apramycin yield of ∆aprl increased from 196 ± 36 mg/L to 2037 ± 198 mg/L, and apramycin disappeared (Fig. 2D and Table S6). To preclude any possible polar effects, we performed ∆aprl complementary. The aprl complementary plasmid pAP605, in which the entire aprl gene is under the control of the PhrD promoter, was introduced by intergenic conjugation into ∆aprl. We screened for the erythromycin-resistance phenotype to isolate the aprl complementary strain, and we designated this strain as ∆aprl::apr1. HPLC analysis showed that the ∆aprl::apr1 restored the ability to produce apramycin (Fig. 2D). Thus, we ruled out the possibility that aprl gene disruption had other polar effects. These results illustrate that Aprl is the 7’-N-methyltransferase in apramycin biosynthesis.

David Crich utilized chemical synthesis methods to prepare a series of apramycin derivatives and investigated their antibacterial activity (Mandhapati et al., 2014). In these derivatives, demethyl-apramycin had good activity against E. coli and Staphylococcus aureus, and functions as good as apramycin in some cases. Because the main product of ∆aprl strain is demethyl-apramycin, this strain provides a convenient, efficient, and controllable method to produce the potentially valuable aminoglycoside antibiotic demethyl-apramycin.

Catalytic Specificity of Aprl
An in vivo gene disruption experiment demonstrated that the aprl gene is involved in the unique methylation process of apramycin synthesis. However, the methylation substrate and mechanism are still unknown. Thus, aprl was cloned into the expression vector pET28a(+), and recombinant Aprl was expressed as an N-terminal His6-tagged protein and expressed in E. coli BL21, as described in Methods. Consisting of 262 amino acids, it is predicted to have a molecular weight of 28.8 KDa. After separation and purification on a nickel column, the molecular size was determined by sodium dodecyl sulfate-polyacrylamide gelelectrophoresis to be consistent with the molecular weight of Aprl (Fig. 3A).

Recombinant Aprl was assayed for the ability to catalyze the SAM-dependent methyl transfer reaction, which converts demethyl-apramycin to apramycin. However, this recombinant protein was not able to catalyze this reaction (Fig. 3C). Thus, it was speculated that the 7’-N methylation of apramycin may occur at the pseudo-trisaccharide instead of the pseudo-tetrasaccharide. We then attempted to hydrolyze demethyl-apramycin to prepare the proper substrate (O’Connor et al., 1976). We identified the product by HPLC and MS and named it demethyl-aprosamine (Fig. S4). Using demethyl-aprosamine as substrate, the catalysis results showed that demethyl-aprosamine was converted into a new compound under the presence of both Aprl and SAM (Fig. 3B). It is worth noting that the reaction product showed a set of two peaks in the HPLC analysis. The splitting peak pattern was also discovered in other reactions such as octuloreumosine heptaacetate biosynthesis of lincomycin A (Sasaki et al., 2012). As the two peaks could not be isolated, we speculated that the two peaks might be mutually convertible isomers. To prepare the authentic aprosamine for HPLC analysis of Aprl enzymatic reaction, apramycin was hydrolyzed. Furthermore, its hydrolysate also showed that a splitting peak was identical with Aprl catalytic product in the HPLC analysis (data not shown). The splitting peak was identified by MS (m/z 379) and NMR (Fig. S5, Fig. S6 and Table S5). Therefore, we concluded that the two new Aprl catalyzed products were aprosamine and its isomer. The results confirm that Aprl catalyze pseudo-trisaccharide methylation rather than pseudo-tetrasaccharide (Fig. 3D).

Methyl groups have stereoelectronic effects on micromolecules and biomacromolecules. As a result, they lead to a diversity of biological effects, containing the selectivity between biological receptors, increasing potency, and protection against enzyme metabolism (Barreiro et al., 2011). Methyltransferases in the biosynthesis of aminoglycoside antibiotics are mainly divided into two types. The first type uses SAM as a methyl donor to catalyze the methylation of the substrate and the other one is a radical SAM enzyme that catalyzes non-universal methylation. In the biosynthesis pathway of the aminoglycoside antibiotic gentamicin, there are four methyltransferases, including GenK, GenD1, GenN, and GenL. (Li et al., 2018). GenK and GenD1 were identified as cobalamin-dependent radical SAM methyltransferases that transfer the methyl group to the carbon atom respectively at the positions of C6- and C4- in gentamicin biosynthesis. They have a common [4Fe-4S] cluster and a highly conserved “CxxxCxxxC” motif in their active sites. The two radical SAM enzymes are reported to extract a hydrogen atom from the corresponding sites of their substrates to provide a radical intermediate with 5’-O-Da originating from SAM. The radical intermediate forms a C-C bond with the methyl group on methylcobalamin to obtain the C-methylated product (Huang et al., 2015; Kim et al., 2013). GenN as the 6’-N-methyltransferase and GenS as the 3’-N-methyltransferase in gentamicin are classified in class I SAM-dependent
Fig. 2 Identification of AprI as the methyltransferase for 7’-N methylation. (A) Schematic representation of the aprI deletion. (B) Verification of ΔaprI mutant by PCR. PCR amplification with wild-type strain chromosome generated a 4.8 kb fragment, and ΔaprI strain chromosome generated a 4.0 kb fragment. (C) The analysis of products by TLC from wild-type S. tenebrarius and the ΔaprI. (D) HPLC analysis was performed by using an evaporative light-scattering detector (ELSD); (i) apramycin standard, (ii) wild-type S. tenebrarius, (iii) ΔaprI, and (iv) ΔaprI containing an aprI-expressing plasmid.
methyltransferase. As the terminal 6′-N-methyltrasferase, GenL has the activity in the conversion of both C2 to C1 and of C1a to C2b. However, GenL is an enzyme with low sequence similarity to GenN and other N-methyltransferases. The reaction mechanism of GenL is still unknown (Li et al., 2018). Similar to AprI, GenN belongs to the HemK family, which has relatively conserved “GxGxG” and “NSPT” sites to interact with S-adenosyl-L-homocysteine. The protein crystal structure of GenN has been determined (Bury et al., 2017). Different from AprI, GenN can catalyze multiple pseudo-trisaccharide substrates, containing A1, A-2, Aε, and A. However, AprI acts on 7′-N of the demethyl-apramycin in this study. As a unique methyltransferase in the biosynthesis of aminoglycoside antibiotics, substrate scopes and reaction mechanisms of AprI deserve further study, and research on this subject will form a basis for the development of new aminoglycoside antibiotics.

Overexpression of aprI by Homologous Recombination in S. tenebrarius Reduced Demethyl-Apramycin as an Impurity in Apramycin Production

Promoter engineering and gene knockout are the most direct and effective metabolic engineering methods for changing metabolic flow. Industrial production of apramycin is performed by microbial fermentation. The commercial apramycin examined in this study (HPLC-ELSD) contains 0.9–1.28% of demethyl-apramycin, which is the major impurity in apramycin biosynthesis (Fig. 2D). However, because there is only one methyl group difference between demethyl-apramycin and apramycin, it is difficult to separate demethyl-apramycin and apramycin in production process. To reduce the production of demethyl-apramycin by metabolic engineering, we decided to increase the expression of aprI. To elevate the genetic stability of engineering strains, we integrated aprI into the chromosome via homologous recombination rather than site-specific integration.

Because S. tenebrarius produces apramycin and carbamyltobramycin simultaneously, disruption of tobramycin biosynthetic pathway not only eliminates the impurity carbamyltobramycin from apramycin, but also turns the shared biosynthetic precursors to apramycin biosynthesis. We attempted to insert the aprI gene in the tobamycin biosynthesis gene cluster to obtain strain for apramycin mono-production. In tobamycin biosynthesis gene cluster of S. tenebrarius, the tobm2 gene was considered as the second glycobytransferase, converting the intermediate 6′-oxo-lividamine to carbamyltobramycin (Fig. 1B) (Park et al., 2011). To confirm tobm2 gene was unrelated to apramycin biosynthesis, the tobM2 gene was disrupted in the wild-type strain of S. tenebrarius. The tobm2 deletion plasmid pAP600 was introduced into S. tenebrarius by conjugation transfer to obtain the tobm2-disruption strain via the double crossover homologous recombination event. The PCR and DNA sequencing results confirmed that the selected strain designated ΔtobM2 does not possess the 1263 bp tobM2 sequence (Fig. S7B). The ΔtobM2 was later cultured to analyze its fermented biosynthetic products. HPLC analysis confirmed that ΔtobM2 strain still produced apramycin and did not produce carbamyltobramycin (Fig. S7C). In addition, semi-quantitative RT-PCR analysis showed that the transcript level of apramycin biosynthesis genes had no significant difference between S. tenebrarius and ΔtobM2 (Fig. S7D).

Next, aprI, which is under the control of PhdB, was inserted into the homology arm of tobM2 via homologous recombination (Fig. 4A and B). The overexpressing strain was named S. tenebrarius IB. Compared to wild-type strain, the demethyl-apramycin yield decreased from 196 ± 36 mg/L to 51 ± 9 mg/L by HPLC assays and the percentage of demethyl-apramycin among the total apramycin products decreased from 1.17% to 0.34% in S. tenebrarius IB. The apramycin yield of the wild-type strain was 2227 ± 320 mg/L and that of S. tenebrarius IB was 2331 ± 210 mg/L. The percentage of apramycin among the total apramycin products increased from 68% to 87% (Fig. 4C and Table S6). Through overexpression aprI, demethyl-apramycin yield further decreased, and the purity of apramycin in the fermentation products was improved. Moreover, the carbamyltobramycin yield of the wild-type strain was 607 ± 111 mg/L and that of S. tenebrarius IB ceased to produce (Fig. 4C and Fig. S8).

We constructed the site-specific integration strain of aprI overexpressing by using plasmid pAP605, which derived from pSET152.
with erythromycin-resistant gene \textit{ermE}, introduced in \textit{S. tenebrarius} to decrease demethyl-apramycin. To determine the stability of the site-specific plasmid, 80 single colonies were randomly selected from each generation, and their genomic DNA was examined by PCR. The results revealed that more than 53.7\% of the colonies eliminated the overexpression plasmid pAP605 from the chromosome after five generations of unselected passage. The homologous recombination strain had better stability compared with the site-specific integration strain, and the stability of \textit{S. tenebrarius} IB was not affected by the passages.

**Conclusions**

AprI was identified as 7'-N-methyltransferase in apramycin biosynthesis by gene disruption and biochemical study. AprI had an activity to transfer a methyl group at the position of 7'-N of demethyl-aprosamine. Through deleting the \textit{aprI} gene, a high-yielding strain of aminoglycoside antibiotic demethyl-apramycin was constructed.

Based on the study of apramycin biosynthetic pathway and tobramycin biosynthetic pathway in \textit{S. tenebrarius}, the \textit{S. tenebrarius} IB with overexpression of \textit{aprI} and disruption of \textit{tobM2} was constructed to decrease the impurity production of demethyl-apramycin and carbamyltobramycin. The content of demethyl-apramycin in the \textit{S. tenebrarius} IB was reduced from 196 ± 36 mg/L to 51 ± 9 mg/L and its rate decreased from 1.17\% to 0.34\%. The carbamyltobramycin titer of the wild-type strain was 607 ± 111 mg/L and that of \textit{S. tenebrarius} IB was null. The high titer and rate of apramycin producing strain was achieved by engineering the biosynthetic pathway of apramycin and tobramycin.

**Fig. 4** Construction and confirmation of the \textit{aprI} overexpressing strain \textit{S. tenebrarius} IB. (A) Schematic representation of the \textit{S. tenebrarius} IB construction via homologous recombination. (B) Verification of \textit{S. tenebrarius} IB homologous recombination events. Primers PhrdB-up and IB-P3 were used and PCR amplification showed that \textit{S. tenebrarius} IB had a 1.2 kb fragment. (C) Analysis of secondary metabolite yields.
In the engineering strain, the titer of apramycin increased from 2227 ± 320 mg/L to 2331 ± 210 mg/L, and the rate increased from 68% to 87%.

**Supplementary Material**

Supplementary material is available online at JIMB (www.academic.oup.com/jimb).

**Authors Contributions**

J. S., H. G., X. N., and H. X. conceived the experiments; J. S. constructed and analyzed mutants; J. S. carried out in vitro analysis; J. S., H. G., Y. L., D. Y., X. N., and H. X. analyzed the results; and H. X., X. N., and J. S. wrote the paper.

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**Competing interests**

The authors declare no competing interests.

**Conflict of Interest**

The authors declare no conflict of interest.

**Data Availability**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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