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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 *apr*I, we identified a new aminoglycoside antibiotic demethyl-apramycin as the main product in *apr*I 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 *apr*I and disruption of the *tob*M2, 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, *apr*I 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). Lividamine is the 3' deoxygenated 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, lividamine is oxidized by AprQ to form 6'-oxo-lividamine, 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'-deoxygenation 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 KNO_3, 0.5 g/L $K_2HPO_4 \cdot 3H_2O$, 0.5 g/L NaCl, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 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

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Fig. 1 Apramycin biosynthetic gene cluster, tobramycin biosynthetic gene cluster and proposed apramycin biosynthetic pathway. (A) Apramycin biosynthetic gene cluster and tobramycin biosynthetic gene cluster. (B) Proposed apramycin biosynthetic pathway.

$MgSO_4{\cdot}7H_2O$, 0.5 g/L $K_2HPO_4{\cdot}3H_2O$, 0.5 g/L NaCl) for mycelium. *S. tenebrarius* and its derivative mutants were cultured in the seed medium (10 g/L soyal bean, 10 g/L glucose, 3 g/L peptone, 1 g/L yeast powder, 5 g/L corn powder, 1 g/L $CaCO_3$) at 37°C for 18 hrs; 3 mL(10% [v/v]) seed culture was used to inoculate the fermentation medium (40 g/L soyal bean, 20 g/L glucose, 20 g/L soluble starch, 5 g/L yeast powder, 5 g/L $CaCO_3$(light), 5 g/L NH_4Cl, 2 g/L $MgSO_4$, 0.05 g/L $FeSO_4$, 0.03 g/L $ZnSO_4$, 0.3 g/L $MnCl_2$) and was incubated for 5 days. *E. coli* strains were maintained in Luria–Bertani (LB) liquid or solid medium (10 g/L yeast extract, 5 g/L tryptone, 10 g/L NaCl) at 37°C with appropriate antibiotic selection at final concentration of 100 μg/mL ampicillin, 25 μg/mL chloramphenicol, and 50 μg/mL kanamycin.

Construction of the *tob*M2-Disruption Plasmids

Based on the aforementioned sequence of the tobramycin biosynthetic gene cluster above, two DNA fragments flanking *tob*M2 were amplified from the genomic DNA of *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 *apr*I-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 *apr*I, two DNA fragments flanking *apr*I were amplified from the genomic DNA of *S. tenebrarius* by using IU-P1/P2 and ID-P3/P4. The PCR products were cloned into pIJ2925 to obtain plasmid pAP601, which was verified by restriction endonuclease digestion and sequencing. Then, the homogenous arm of *apr*I was cloned into pSPU310 to obtain the gene disruption plasmid pAP602.

Targeted Gene Deletion

To create double-cross deletion mutants Δ*tob*M2 and Δ*apr*I 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 Δ*apr*I Mutant Strain

For complementation of the *apr*I gene mutant, pAP603 was constructed as the intermediate vector by inserting *apr*I into pHJK241 (Gao et al., 2017) between the *Nco*I and *Hind*III sites. Then pAP605 was constructed by inserting *apr*I into the vector pEAP1 (Gu et al., 2015) under the control of the constitutive promoter P*hrd*B. The plasmid pAP605 was then introduced into Δ*apr*I through conjugation. Complemented exconjugants were verified on agar medium containing erythromycin (100 μg/mL) and confirmed by PCR.

Construction of the *apr*I Overexpressing Strain Based on Homologous Recombination

The fragment of P*hrd*B, *apr*I, and T0 was digested from pAP603 by *Bgl*II, 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 H_2SO_4 and agitated 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 NH_4OH. This pretreated supernatant was then centrifuged at 7000 r/min for 10 min. The supernatant was readjusted to pH 5.5 with H_2SO_4, applied onto a strongly acidic resin 001 × 7 (Anhui Sanxing Resin Technology), and then the bound substances were eluted with 2 mol/L NH_4OH. The eluate was used for bioassays and thin-layer chromatography analysis (TLC). Propanol–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 a LC-10AT system with an evaporative light scattering detector (ELSD). Reverse C_{18} 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 *apr*I 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. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) data were recorded on a Bruker AV600 at a frequency of 600 MHz using D_2O 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 *apr*I 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 *Nde*I and *Eco*RI 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 isopropylthiogalactoside (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 K_2HPO_4/KH_2PO_4 (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 Δ*tob*M2 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 transcripted using the PrimeScript^{TM} 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 *tob*M2 were tested.

Result and Discussion

Identify Function of *apr*I by Gene Deletion and Complementation

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

To clarify the function of *apr*I, *apr*I 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 *apr*I mutant strain (Δ*apr*I) generated a 4.0 kb fragment because of a 789 bp deletion (Fig. 2A and B). The 4.0 kb fragment amplified from Δ*apr*I was further confirmed by DNA sequencing (data not shown). The results verified that *apr*I was successfully disrupted in Δ*apr*I. We cultured the Δ*apr*I 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 Δ*apr*I produced a new bioactive product 2 instead of apramycin (Fig. 2C). HPLC analysis confirmed that Δ*apr*I 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 Δ*apr*I 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 Δ*apr*I. The ^{13}C-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 Δ*apr*I 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 Δ*apr*I complementary. The *apr*I complementary plasmid pAP605, in which the entire *apr*I gene is under the control of the P*hrd*B promotor, was introduced by intergenic conjugation into Δ*apr*I. We screened for the erythromycin-resistance phenotype to isolate the *apr*I complementary strain, and we designated this strain as Δ*apr*I::*apr*I. HPLC analysis showed that the Δ*apr*I::*apr*I restored the ability to produce apramycin (Fig. 2D). Thus, we ruled out the possibility that *apr*I gene disruption had other polar effects. These results illustrate that AprI 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 Δ*apr*I strain is demethyl-apramycin, this strain provides a convenient, efficient, and continuable method to produce the potentially valuable aminoglycoside antibiotic demethyl-apramycin.

Catalytic Specificity of AprI

An *in vivo* gene disruption experiment demonstrated that the *apr*I gene is involved in the unique methylation process of apramycin synthesis. However, the methylation substrate and mechanism are still unknown. Thus, *apr*I was cloned into the expression vector pET28a(+), and recombinant AprI was expressed as an N-terminal His_6-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 AprI (Fig. 3A).

Recombinant AprI 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 AprI 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 octulofuremose 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 AprI enzymatic reaction, apramycin was hydrolyzed. Furthermore, its hydrolysate also showed that a splitting peak was identical with AprI 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 AprI catalyzed products were aprosamine and its isomer. These results confirm that AprI 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 "CxxxCxxC" 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'-dAdo 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). GenL as the 6'-*N*-methyltranserase and GenN as the 3''-*N*-methyltranserase in gentamicin are classified in class I SAM-dependent

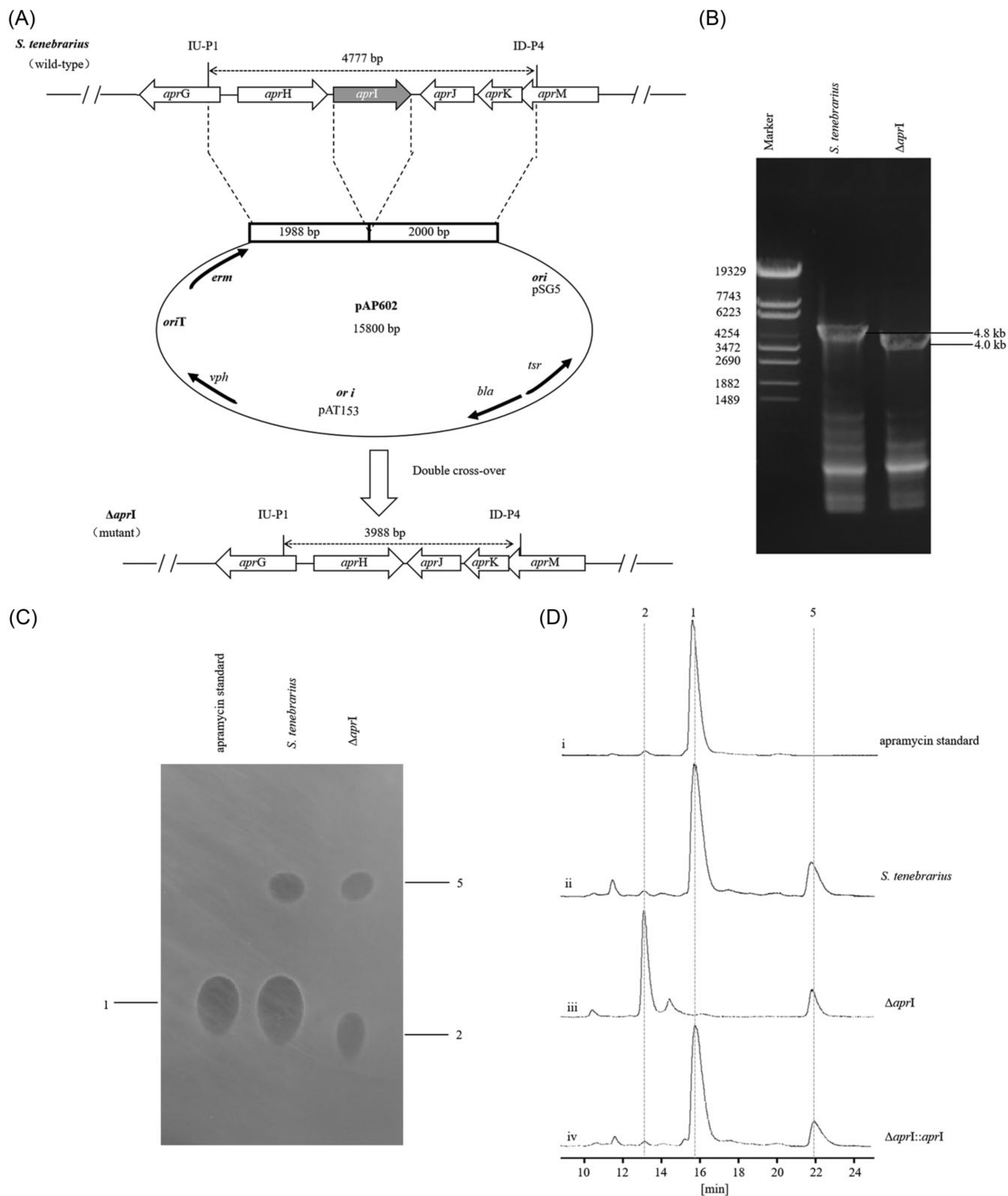

Fig. 2 Identification of AprI as the methyltransferase for 7'-*N* methylation. (A) Schematic representation of the *apr*I deletion. (B) Verification of Δ*apr*I mutant by PCR. PCR amplification with wild-type strain chromosome generated a 4.8 kb fragment, and Δ*apr*I strain chromosome generated a 4.0 kb fragment. (C) The analysis of products by TLC from wild-type S. *tenebrarius* and the Δ*apr*I. (D) HPLC analysis was performed by using an evaporative light-scattering detector (ELSD); (i) apramycin standard, (ii) wild-type S. *tenebrarius*, (iii) Δ*apr*I, and (iv) Δ*apr*I containing an *apr*I-expressing plasmid.

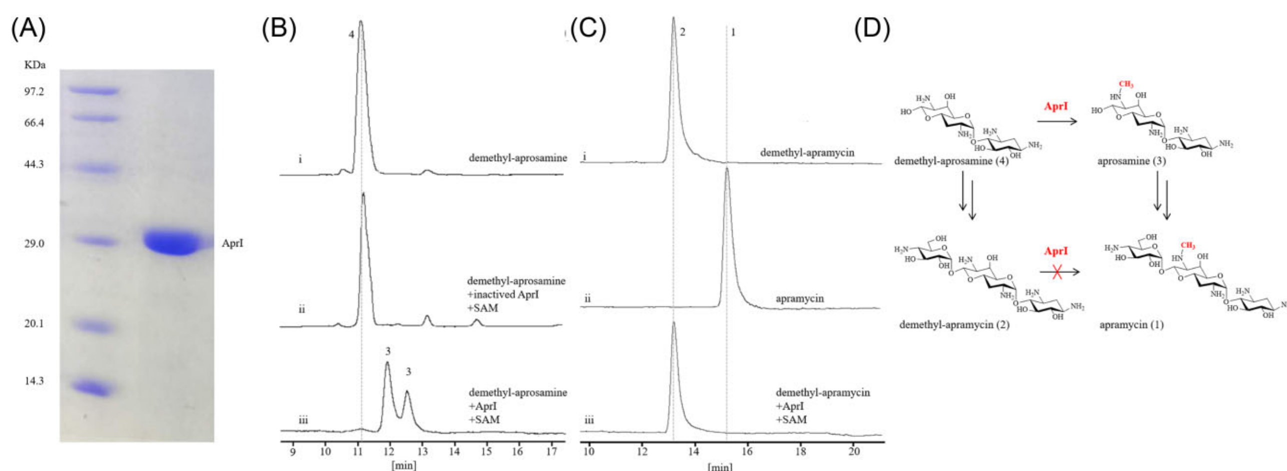

Fig. 3 Characterization of AprI as a SAM-dependent methyltransferase. (A) SDS-PAGE analysis of AprI. (B) HPLC-ELSD analysis of the conversion of demethyl-aprosamine by AprI *in vitro*; (i) demethyl-aprosamine, (ii) control reaction in which AprI was omitted, and (iii) AprI reaction with demethyl-aprosamine and SAM. AprI converted demethyl-aprosamine into aprosamine. (C) HPLC-ELSD analysis of the conversion of demethyl-apramycin by AprI *in vitro*. (i) demethyl-apramycin, (ii) apramycin, and (iii) AprI reaction with demethyl-apramycin and SAM. AprI could not convert demethyl-apramycin into apramycin. (D) The 7'-*N* position methylation biosynthetic route of apramycin. AprI converts demethyl-aprosamine into aprosamine. However, AprI cannot convert demethyl-apramycin into apramycin.

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, Ae, and A. However, AprI acts on 7'-*N* of the demethyl-aprosamine 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 *apr*I 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 *apr*I. To elevate the genetic stability of engineering strains, we integrated *apr*I 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 *apr*I gene in the tobramycin biosynthesis gene cluster to obtain strain for apramycin mono-production. In tobramycin biosynthesis gene cluster of *S. tenebrarius*, the *tob*M2 gene was considered as the second glycosyltransferase, converting the intermediate 6'-oxo-lividamine to carbamyltobramycin (Fig. 1B) (Park et al., 2011). To confirm *tob*M2 gene was unrelated to apramycin biosynthesis, the *tob*M2 gene was disrupted in the wild-type strain of *S. tenebrarius*. The *tob*M2 deletion plasmid pAP600 was introduced into *S. tenebrarius* by conjugation transfer to obtain the *tob*M2-disruption strain via the double crossover homologous recombination event. The PCR and DNA sequencing results confirmed that the selected strain designated Δ*tob*M2 does not possess the 1263 bp *tob*M2 sequence (Fig. S7B). The Δ*tob*M2 was later cultured to analyze its fermented biosynthetic products. HPLC analysis confirmed that Δ*tob*M2 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 Δ*tob*M2 (Fig. S7D).

Next, *apr*I, which is under the control of P*hrd*B, was inserted into the homology arm of *tob*M2 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 *apr*I, 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 *apr*I overexpressing by using plasmid pAP605, which derived from pSET152

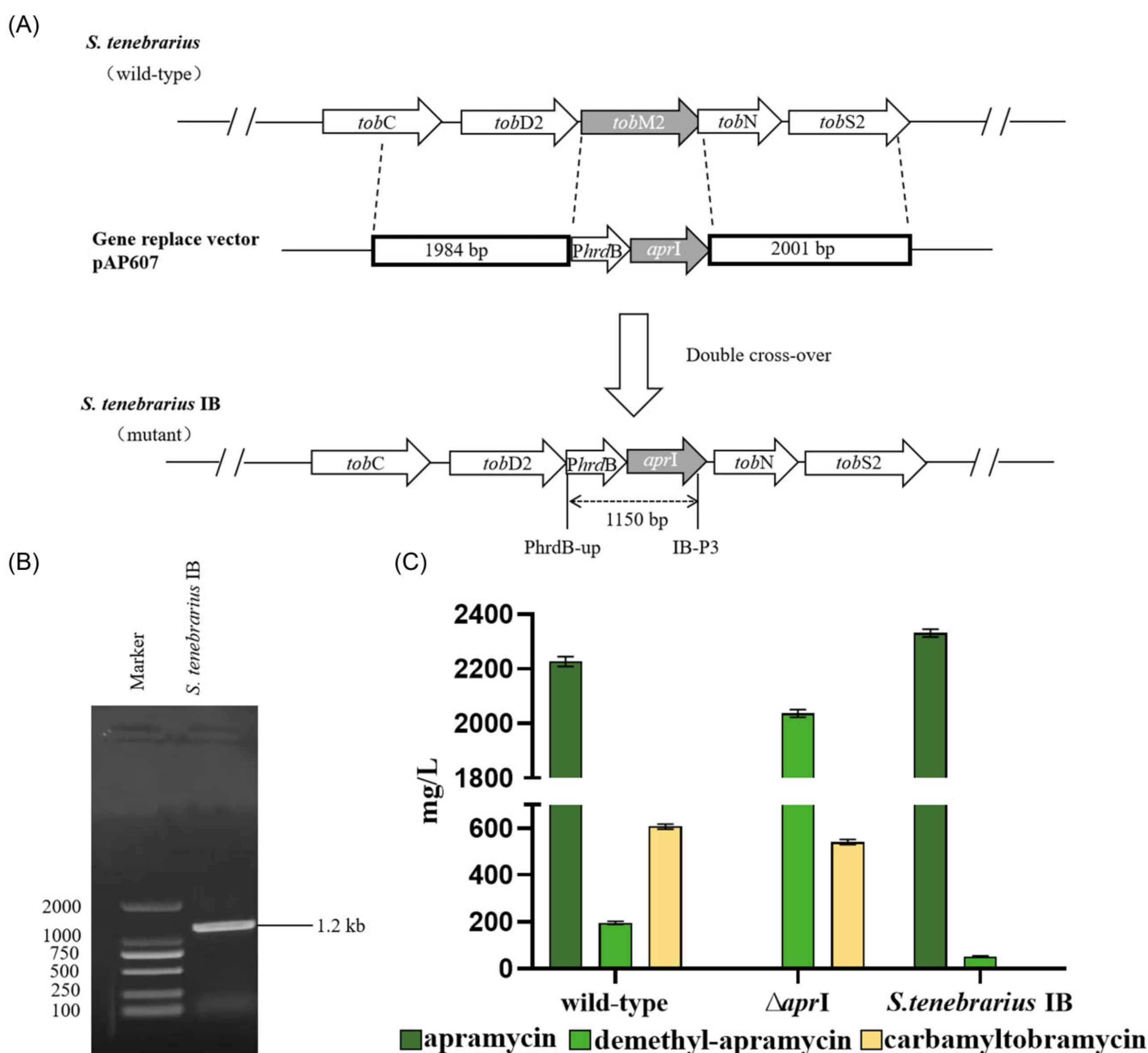

Fig. 4 Construction and confirmation of the *apr*I overexpressing strain S. *tenebrarius* IB. (A) Schematic representation of the S. *tenebrarius* IB construction via homologous recombination. (B) Verification of S. *tenebrarius* IB homologous recombination events. Primers *Phrd*B-up and IB-P3 were used and PCR amplification showed that S. *tenebrarius* IB had a 1.2 kb fragment. (C) Analysis of secondary metabolite yields.

with erythromycin-resistant gene *erm*E, introduced in 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 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 *apr*I 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 S. *tenebrarius*, the S. *tenebrarius* IB with overexpression of *apr*I and disruption of *tob*M2 was constructed to decrease the impurity production of demethyl-apramycin and carbamyltobramycin. The content of demethyl-apramycin in the 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 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.

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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