Characterization of Methyltransferase from Apramycin Biosynthetic Gene Cluster and Improvement Production of Apramycin in Engineered *Streptoalloteichus Tenebrarius*

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Keywords: *Streptoalloteichus tenebrarius*, apramycin biosynthesis pathway, aprI gene, methyltransferase

Posted Date: September 30th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-927201/v1>

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Abstract

Background

Apramycin is a structurally unique aminoglycoside, used in veterinary medicine or the treatment of *Salmonella*, *Escherichia coli* and *Pasteurella multocida* infections in farm. Although discovered and used many years ago, many biosynthetic steps of apramycin are still obscure.

Results

In this study, we identified a HemK family methyltransferase, *aprI*, involved in apramycin biosynthesis. The function of *aprI* was studied by using gene disruption and biochemical experiments, and a new aminoglycoside antibiotic demethyl-apramycin was purified from *aprI* disruption strain. Experiments proved that AprI converted demethyl-aprosamine to aprosamine *in vitro*. Based on this, the apramycin production strain was improved by overexpression the AprI to decrease the impurity production.

Conclusions

We have identified *aprI* is a 7'-N-methyltransferase gene in apramycin biosynthesis and confirmed the substrate of methyltransferase. Engineering of *aprI* resulted in a strain producing a new aminoglycoside demethyl-apramycin and apramycin mono-producing strain with less impurity production. Finally, the yield of demethyl-apramycin in apramycin mono-producing strain decreased from 196±36 mg/L to 51±9 mg/L, and the yield of apramycin increased from 2227±320 mg/L to 2331±210 mg/L.

Introduction

Apramycin, produced by *Streptoalloteichus tenebrarius*, is a structurally unique aminoglycoside antibiotic.¹ As frequently employed [veterinary antibiotic drug](#), apramycin has the high antimicrobial activity on important gram-negative pathogens and has been mainly used for the treatment of *Salmonella*, *Escherichia coli* and *Pasteurella multocida* infections in poultry, swine or bovine.²⁻³ Meanwhile, apramycin is a potent antibacterial with low ototoxicity, which properties distinct from all human used aminoglycosides. The properties attracted the attention of many research teams.⁴⁻⁵

Apramycin, carbamyltobramycin and carbamylkanamycin B are isolated from *S. tenebrarius*.⁶ They all belong to 2-dexyostreptomycin (2-DOS) aminoglycosides. Apramycin is characterized by 2-DOS linked with 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).⁷ Apramycin has independent biosynthetic gene cluster (GenBank accession number: AJ629123) (Figure 1A). Base on bioinformatical analysis, the genes for the common intermediates 2-deoxystreptamine and paromamine can be easily identified both in apramycin and tobramycin biosynthetic gene clusters.⁸ Lividamine is the 3' deoxygenated product of paromamine

under the function of AprD3 and AprD4.⁹⁻¹² Then, lividamine was oxidized by AprQ to form 6'-oxo-lividamine which may be the intermediate of bicyclic 3'-deoxyoctose moiety.¹³ Besides, it was confirmed that AprD3 and AprD4 are responsible for carbamyltobramycin 3'-deoxygenation process (Figure 1B).¹⁴ However, the biosynthetic pathway of the unique structure of apramycin is still obscure. Here, we report the identification and characterization of a 7'-N-methyltransferase gene, *aprl*, and its protein substrate, demethyl-aprosamine, involved in the conversion.

As other antibiotics, the accumulation of intermediate metabolites is also observed in apramycin fermentation products. The final apramycin products contain 0.9-2.7% of demethyl-apramycin. Therefore, demethyl-apramycin is one of the main impurity, its production should be controlled. Based on the study of the biosynthetic pathways of apramycin and tobramycin, we metabolic engineering *S. tenebrarius* to optimize apramycin production through block tobramycin biosynthesis and decrease impurity production.

Results And Discussion

Identify function of *aprl* by gene deletion and complementation. Bioinformatically analyze the apramycin biosynthetic gene cluster showed that Aprl possessed homology with known methyltransferase, sharing a high degree of sequence identity with the N-methyltransferase CalM of *Streptomyces chartreusis* (35%)¹⁸ and a lower degree of sequence identity with the 3"-N-methyltransferase GenN of *Micromonospora echinospora* (29%).¹⁹ All of them belong to the HemK superfamily, which catalyzes methyl group transfer of a variety of substrates. Thus, we deduce Aprl is the 7'-N-methyltransferase in apramycin biosynthesis. To clarify this hypothesis, we carried out both genetic and biochemical analysis of Aprl in this work.

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 ($\Delta aprl$) generated a 4.0 kb fragment because of a 789 bp deletion (Figure 2A and Figure 2B). The 4.0 kb fragment amplified from $\Delta aprl$ was further confirmed by DNA sequencing (data not shown). The results verified that *aprl* was successfully disrupted in $\Delta aprl$. The $\Delta aprl$ was cultured to analyze its fermented products. The wild-type strain of *S. tenebrarius* was used as control under the same fermentation conditions. Compared to the wild-type strain, the total antibiotic production in the $\Delta aprl$ decreased from 2620 mg/L to 2183 mg/L (measured by bioassay). TLC coupled with an antimicrobial activity assay showed the $\Delta aprl$ produced a new bioactive product instead of apramycin (Figure 2C). HPLC analysis confirmed that $\Delta aprl$ accumulated a new compound 2 (Figure 2D). By HRMS analysis, the new component 2 produced by $\Delta aprl$ was considered as demethyl-apramycin (Figure S1). The demethyl product was also analyzed by MS/MS (Figure S1). 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 379,²⁰ suggesting the demethyl-apramycin might lack a methyl group at 7'-N. 2 then was purified from the fermentation broth of $\Delta aprl$. The ¹³C-NMR analysis definitively confirmed that compound 2 lacked a 7'-N-methyl group (Figure S2 and Table S4). To preclude any possible of polar

effects, we performed $\Delta aprI$ complementary. The *aprI* complementary plasmid pAP605, in which the entire *aprI* gene is under the control of the *PhrdB* promoter, was introduced by intergenic conjugation into $\Delta aprI$. We screened for the erythromycin-resistance phenotype to isolate the *aprI* complementary strain, and we designated this strain as $\Delta aprI::aprI$. HPLC analysis showed that the $\Delta aprI::aprI$ restored the ability to produce apramycin (Figure 2D). Thus, the possibility that *aprI* gene disruption had other polar effects can be ruled out. These results illustrate that AprI was 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. In these derivatives, demethyl-apramycin had good activity against *E.coli* and *Staphylococcus aureus*, and was as good as apramycin in some cases.²¹ As the main product of $\Delta aprI$ strain is demethyl-apramycin, this strain provide a convenience, efficient and continuable method for production the potentially valuable aminoglycoside antibiotic demethyl-apramycin.

Catalytic specificity of AprI. An *in vivo* gene disruption experiment demonstrated that the *aprI* gene is involved in the unique methylation process of apramycin synthesis. However, the methylation substrate and mechanism are still unknown. Thus, *aprI* was cloned into the expression vector pET28a(+), and recombinant AprI was expressed as an N-terminal His₆-tagged protein and expressed in *E. coli* BL21 as described in Methods. It is composed of 262 amino acids and 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 SDS-PAGE to be consistent with the molecular weight of AprI (Figure 3A).

Recombinant AprI was assayed for the ability to catalyze the SAM-dependent methyl transfer reaction which convert demethyl-apramycin to apramycin. However, this recombinant protein was not able to catalyze this reaction (Figure 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.²² We identified the product by HPLC and MS and named it demethyl-aprosamine (Figure S3). 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 (Figure 3B). It is worth noting that the reaction product showed two peaks in the HPLC analysis. As the two peaks cannot 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 two peaks is identical with AprI catalytic product in the HPLC analysis (data not shown). Therefore, the two new AprI catalyzed products together were deduced as aprosamine and its isomer (Figure S4 and Figure S5). These results confirm that AprI catalyze pseudo-trisaccharide methylation rather than pseudo-tetrasaccharide (Figure 3D).

Methyl groups have stereoelectronic effects on micromolecules and biomacromolecules, thereby leading to a diversity of biological effects, containing the selectivity between biological receptors, increased potency, and protection against enzyme metabolism.²³ Methyltransferases in the biosynthesis of

aminoglycoside antibiotics are mainly divided into two types. The first type is using SAM as a methyl donor to catalyze the methylation of the substrate. And the other is radical SAM enzyme catalyzing non-universal methylation. In the biosynthesis pathway of the aminoglycoside antibiotic gentamicin, there are four methyltransferases, including GenK, GenD1, GenN, and GenL.²⁴ GenK and GenD1 were identified as cobalamin-dependent radical SAM methyltransferases that transfer the methyl group to the carbon atom at the position of C6' and C4" in gentamicin biosynthesis, respectively. 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 utilizing 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.²⁵⁻²⁶ GenL as the 6'-N-methyltransferase and GenN as the 3"-N-methyltransferase in gentamicin are classified in class I SAM-dependent methyltransferase. As the terminal 6'-N-methyltransferase, GenL has the activity in the conversion of both C2 to C1 and of C1a to C2b. But GenL is an enzyme with low sequence similarity to GenN and other N-methyltransferases, reaction mechanism of GenL still unknown.²⁴ Similar to AprI, GenN belongs to the HemK family, which has relatively conserved "GXGXG" and "NSPT" sites to interact with SAH. The protein crystal structure of GenN has been determined.²⁷ Different from AprI, GenN can catalyze multiple pseudo-trisaccharide substrates, containing A1, A-2, Ae, and A. However, AprI act on 7'-N of the demethyl-apramycin in this study. As a unique site methyltransferase in the biosynthesis of aminoglycoside antibiotics, substrate scopes and reaction mechanisms of AprI deserve further study and its research will provide 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. Our final products contain 0.9-2.7% of demethyl-apramycin, which is the major impurity in apramycin biosynthesis. However, there is only a methyl group difference between demethyl-apramycin and apramycin, it is pretty difficult to separate demethyl-apramycin and apramycin in production process. To reduce the production of demethyl-apramycin by metabolic engineering, we intended 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* produce apramycin and tobramycin simultaneously, disruption tobramycin biosynthetic pathway not only can eliminate the impurity tobramycin from apramycin, but also tuned the shared biosynthetic precursors to apramycin biosynthesis. We attempted to insert the *aprI* gene in the tobramycin biosynthesis gene cluster to obtain strain for apramycin mono-production. In tobramycin biosynthesis gene cluster of *S. tenebrarius*, the *tobM2* gene was considered as the second glycosyltransferase, converting the intermediate nebramine to tobramycin (Figure 1B).¹⁴ 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 Δ *tobM2* does not possess the 1263 bp *tobM2* sequence. The Δ *tobM2* was later cultured to analyze its fermented biosynthetic products. HPLC analysis confirmed that Δ *tobM2* strain still produced apramycin and did not produce tobramycin (Figure S6).

Next, *aprI* which are under the control of *PhrdB*, was inserted into the homology arm of *tobM2* via homologous recombination (Figure 4A and Figure 4B). The overexpressing strain was named *S. tenebrarius* IB. HPLC assays demonstrated that the demethyl-apramycin yield decreased from 196±36 mg/L to 51±9 mg/L and that the percentage of demethyl-apramycin among the total apramycin products decreased from 1.17% to 0.34%. The apramycin yield of the wild-type strain was 2227±320 mg/L and that of *S. tenebrarius* IB was 2331±210 mg/L (Figure 4C). Through overexpression *aprI*, demethyl-apramycin yield was further decreased, and the purity of apramycin in the fermentation products was improved. Moreover, the tobramycin yield of the wild-type strain was 607±111 mg/L and that of *S. tenebrarius* IB was no more producing (Figure 4C).

In the process of constructing *aprI* overexpressing strain, we attempted to obtain the [anticipated](#) strain using site-specific integration plasmid pAP605. However, [previous research](#) had suggested that successive passages of strains lead to elimination of the site-specific integration plasmid.¹⁵ We compared the site-specific integration and the homologous recombination by comparing the products through five passages. The homologous recombination strain had better stability compared with the site-specific integration strain, and the production of *S. tenebrarius* IB is not affected by the passages (data not shown).

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. By deletion the *aprI* gene, an aminoglycoside antibiotic demethyl-apramycin high producing strain was constructed. Based on the study of apramycin biosynthetic pathway and tobramycin biosynthetic pathway in *S. tenebrarius*, the strain overexpression of *aprI* was constructed to decrease the impurity production of demethyl-apramycin and tobramycin. The content of demethyl-apramycin in the *aprI* overexpression strain was reduced from 196±36 mg/L to 51±9 mg/L. And apramycin in the *aprI* overexpression strain was increased from 2227±320 mg/L to 2331±210 mg/L. The tobramycin yield of the wild-type strain was 607±111 mg/L and that of *aprI* overexpression strain was no more producing.

Materials And Methods

Strains and growth conditions. The plasmids used in this study are listed in Table S1. The strains used in this study are listed in Table 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 *Streptoalloteichus 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 MgSO₄·7H₂O, 0.5 g/L K₂HPO₄·3H₂O, 0.5 g/L NaCl) for mycelium. *S. tenebrarius* and its derivative mutants were cultured in the seed medium (10 g/L soya bean, 10 g/L glucose, 3 g/L peptone, 1 g/L yeast powder, 5 g/L corn powder, 1 g/L CaCO₃) at 37°C for 18 hours, 3 mL (10% [v/v]) seed culture was used to inoculate the fermentation medium (40 g/L soya bean, 20 g/L glucose, 20 g/L soluble starch, 5 g/L yeast powder, 5 g/L CaCO₃(light), 5 g/L NH₄Cl, 2 g/L MgSO₄, 0.05 g/L FeSO₄, 0.03 g/L ZnSO₄, 0.3 g/L MnCl₂) 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 *tobM2*-disruption plasmids. Base on the sequence of the tobramycin biosynthetic gene cluster above, two DNA fragments flanking *tobM2* were amplified from the genomic DNA of *S. tenebrarius*¹⁵ by using M2U-P1/P2 and M2D-P3/P4. The PCR products were fusion 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 *aprl*-disruption plasmids. The sequence of the apramycin biosynthetic gene cluster has been deposited in the NCBI under the accession number GenBank AJ629123. For deletion of the apramycin biosynthesis gene *aprl*, two DNA fragments flanking *aprl* 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 *aprl* was cloned into pSPU310 to obtain the gene disruption plasmid pAP602.

Targeted gene deletion. To create double-cross deletion mutants Δ *tobM2* and Δ *aprl* based on wild-type strain *S. tenebrarius*, the corresponding plasmids pAP600 and pAP602 were introduced into *S. tenebrarius* by conjugation, respectively, and mutants screening were carried out using the same method described before.⁷ The desired deletion mutants were identified by PCR using the checking primers.

Complementation of Δ *aprl* mutant strain. For complementation of the *aprl* gene mutant, pAP603 was constructed as the intermediate vector by inserting *aprl* into pHJK241¹⁶ between the *Nco*I and *Hind*III sites. Then pAP605 was constructed by inserting *aprl* into the vector pEAP1¹⁷ under the control of the constitutive promoter *PhrdB*. The plasmid pAP605 was then introduced into Δ *aprl* by conjugation. Complemented exconjugants were verified on agar medium containing erythromycin (100 µg/mL) and confirmed by PCR.

Construction of the *aprl* overexpressing strain based on homologous recombination. The fragment of *PhrdB*, *aprl* and T0 was digested from pAP603 by *Bgl*II, and then was added to plasmid pAP600 to

obtain the recombinated plasmid pAP607. Plasmid pAP607 was introduced into *S. tenebrarius* by 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 agitated for 1 hour. The acidified broth was centrifuged at 7000 r/min for 10 minutes, 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 minutes. 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). Propanol–methanol–25% ammonium hydroxide (20:25:23) was used for the solvent system of TLC. The bioassay was performed with *Bacillus subtilis* by 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 C₁₈ column (4.6250 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. 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 ESI-MS 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 using pfu polymerase (BBI) and the oligonucleotides (Takara). The PCR product was purified by gel extraction, and inserted into vector pET-28a (+) between the *NdeI* and *EcoRI* sites to create pAP606. The resulting construct was verified by DNA sequencing, then 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, then the cultures 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 minutes, and 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 minutes on ice. Supernatant was separated from cell debris by centrifugation at 12000 r/min for 40 minutes at 4°C and passed through a 1 mL IMAC 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 μ L total reaction volume. The reaction was incubated at 30°C and then stopped by the addition of 1x volume of chloroform, followed by vigorous vortexing to denature the proteins. The mixture was centrifuged at 10,000 r/min for 5 minutes, and the supernatant was subjected to HPLC-ELSD and MS analysis.

Abbreviations

2-DOS: 2-dexoystreptomycin

SAM: S-adenosylmethionine

TLC: Thin-layer chromatography analysis

HPLC: High-performance liquid chromatography

ELSD: Evaporative light scattering detector

NMR: Nuclear magnetic resonance

Declarations

Availability of data and materials

The data that support the findings of this study are available from the corresponding author, upon request.

Acknowledgments

Not applicable.

Funding

This work was supported by the Scientific Research Fund of Liaoning Provincial Education Department (2017LZD05, 2019LZD03).

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Authors Contributions

J. S., H. G., X. N., and H. X. conceived the experiments; and 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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Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Figures

Figure 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.

Figure 2

Identification of AprI as the methyltransferase for 7'-N methylation. (A) Schematic representation of the *aprI* deletion. (B) Verification of $\Delta aprI$ mutant by PCR. PCR amplification with wild-type strain chromosome generated a 4.8 kb fragment, and $\Delta aprI$ strain chromosome generated a 4.0 kb fragment. (C) The analysis of products by TLC from wild-type *S. tenebrarius* and the $\Delta aprI$. (D) HPLC analysis was

performed by using an evaporative light-scattering detector (ELSD); (i) apramycin standard, (ii) wild-type *S. tenebrarius*, (iii) Δ AprI, and (iv) Δ AprI containing an aprI-expressing plasmid.

Figure 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 convert 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 cannot convert demethyl-apramycin into apramycin. (D) The 7'-N position methylation biosynthetic route of apramycin.

Figure 4

Construction and confirmation of the *apri* 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 PhrdB-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.

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