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Characterization of a 3-hydroxyanthranilic acid 6-hydroxylase involved in paulomycin biosynthesis

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ABSTRACT

Paulomycins (PAUs) refer to a group of glycosylated antibiotics with attractive antibacterial activities against Gram-positive bacteria. They contain a special ring A moiety that is prone to dehydrate between C-4 and C-5 to a quinone-type form at acidic condition, which will reduce the antibacterial activities of PAUs significantly. Elucidation of the biosynthetic mechanism of the ring A moiety may facilitate its structure modifications by combinatorial biosynthesis to generate PAU analogues with enhanced bioactivity or stability. Previous studies showed that the ring A moiety is derived from chorismate, which is converted to 3-hydroxyanthranilic acid (3-HAA) by a 2-amino-2-deoxyisochorismate (ADIC) synthase, a 2,3-dihydro-3-hydroxyanthranilic acid (DHHA) synthase, and a DHHA dehydrogenase. Unfortunately, little is known about the conversion process from 3-HAA to the highly decorated ring A moiety of PAUs. In this work, we characterized Pau17 as an unprecedented 3-HAA 6-hydroxylase responsible for the conversion of 3-HAA to 3,6-DHAA by *in vivo* and *in vitro* studies, pushing one step forward toward elucidating the biosynthetic mechanism of the ring A moiety of PAUs.

1. Introduction

Paulomycins (PAUs A-F) isolated from *Streptomyces paulus* strain 273 and several other *Streptomyces* strains exhibit good antibacterial activities against a variety of Gram-positive bacteria and have potential use to treat urethritis and *chlamydia* infections [1–3]. Structurally, PAUs are a group of glycosylated antibiotics with a central acetyl-allose moiety connecting with a quinone-like ring A, an isothiocyanate containing paulic acid, and a C-4″ hydroxyethyl branched paulomycose decorated by various fatty acyl chains (Fig. 1A) [1,4]. In addtion, PAU G, a PAU analogue exhibiting strong cytotoxic activities was isolated from a marine bacterium *Micromonospora matsumotoense* M − 412, which lacks the paulomycose moiety but has a 3′-acetyl group at its 6-deoxy-allose moiety (Fig. 1A) [5]. Notably, the ring A of PAUs is a quite special quinone-like structure with a saturated C-4 and a *C*-glycosidic bond and a hydroxyl group at C-5. PAUs are prone to dehydrate between C-4 and C-5 to form a quinone-type ring A, which will reduce their antibacterial activities significantly [6].

Recently, three PAUs biosynthetic gene clusters from *S. paulus* NRRL 8115, *Streptomyces albus* J1074 and *Streptomyces* sp. YN86 were identified [4,7]. Although the biosynthetic logic of the paulic acid moiety is still a mystery, some progresses have been made in understanding PAU biosynthesis of the other three parts by *in vivo* and *in vitro* investigations. It was proposed that PAUs are installed in a convergent model. The C-6′ acetylation of the allose moiety is catalyzed by Pau24 (Plm22) and the branched octose, paulomycose, is from NDP-hexose derived from glucose-1-phosphate. After being loaded to the C-3′ of the allose moiety, the C-4″ acetyl branch of the octose undergoes a stereospecific ketoreduction catalyzed by Pau7 (Plm4) and then a fatty acyl decoration by Pau6 (Plm3) to shape the paulomycose moiety [7,8].

The ring A of PAUs was proposed to be derived from chorismate, which is converted to 2,3-dihydro-3-hydroxyanthranilic acid (DHHA) by two steps analogous to the phenazine biosynthetic pathway. Chorismate is firstly converted to 2-amino-2-deoxyisochorismate (ADIC) by Pau18 (Plm15), then to DHHA by Pau19 (Plm16) [4,7]. Recently, we showed that Pau20 (Plm17) is a DHHA dehydrogenase that converts DHHA to 3-hydroxyanthranilic

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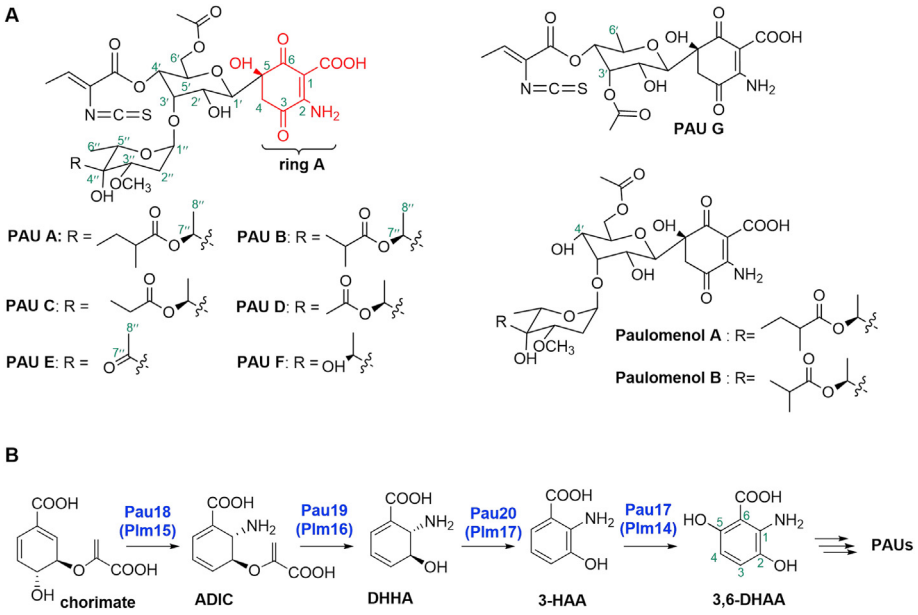


Fig. 1. Structures of PAUs (A) and the proposed biosynthetic pathway for the ring A moiety of PAUs (B).

acid (3-HAA) and constructed a third *de novo* biosynthesis pathway for nicotinamide adenine dinucleotide (NAD^+) based on this discovery (Fig. 1B) [9]. The feeding experiments to *S. paulus* mutant strains confirmed that both DHHA and 3-HAA are intermediates of PAU biosynthesis. Unfortunately, little is known about the conversion process from 3-HAA to the highly decorated PAU ring A.

Based on the structures of 3-HAA and the ring A moiety, we proposed that at least two hydroxylation and one *C*-glycosylation steps are needed for the conversion of 3-HAA to ring A. In this study, we characterized Pau17 as a 3-HAA 6-hydroxylase involved in the ring A formation. In addition, Pau17 displayed certain substrate promiscuity and could catalyze the hydroxylation of a 3-HAA analogoue, 2,3-dihydroxybenzoic acid (2,3-DHBA).

2. Materials and methods

2.1. Bacterial strains, media and plasmids

Strains and plasmids used in this study are summarized in Table 1. *E. coli* JM109 was used for general DNA cloning. *E. coli* BL21 was used for protein preparing. *E. coli* ET12567/pUZ8002 was used for *E. coli-Streptomyces* conjugation. All *E. coli* strains were incubated in Luria-Bertani (LB) medium at 37 °C. *S. paulus* NRRL 8115 and its mutants were cultured on mannitol/soya (MS) agar at 28 °C for sporulation. For metabolism analysis, *Streptomyces* strains were grown in GS-7 medium for seed culture and R5α medium for PAU production [10,11]. The concentrations of antibiotics were as follows if needed: kanamycin, 50 μg/mL; apramycin, 50 μg/mL; and nalidixic acid, 25 μg/mL.

2.2. DNA manipulation and sequence analysis

DNA sequencing was performed in Biosune (Shanghai, China). PCRs were performed with KOD-Plus DNA polymerase (Toyobo, Osaka, Japan) or Taq DNA polymerase (TransGene, Beijing, China). Restriction enzyme digestions, ligations and transformations were carried out following general methods [12]. *E. coli-Streptomyces* conjugations were performed as described [13]. A BLASTP search was used to find protein homologs (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Protein sequences alignments were performed using

Table 1
Strains and plasmids used in this study.

Strains or plasmids	Characteristics[a]	Source
Escherichia coli		
JM 109	General cloning host	Lab stock
BL21(DE3)	Host for protein expression	Novagen
ET12567/pUZ8002	Strain for intergeneric conjugation	Invitrogen
Streptomyces		
S. paulus NRRL 8115	Wild-type strain	NRRL
S. paulus Δ*pau17*	*S. paulus* NRRL 8115 *pau17*::*aph* mutant	This work
S. paulus com-*pau17*	*S. paulus* Δ*pau17* harboring pSET152-*pau17*	This work
Plasmids		
pUC119::KanR	Amp^r, Kan^r, replicating vector in *E. coli*	[28]
pKC1132	Apr^r, *E. coli-Streptomyces* shuttle vector	[13]
pSET152::ermE*	*E.coli-Streptomyces* shuttle vector, with *ermE** promoter	[29]
pET28a	Kan^r, protein production vector	Novagen
pKC1132-*pau17*	Kan^r, pKC1132 harboring *aph* cassette and homologous sequences flanking *pau17*	This work
pSET152-*pau17*	Apr^r, pSET152::ermE* with *pau17* under the control of *ermE**	This work
pET28a-*pau17*	pET28a derived, for expression of the *pau17* gene	This work

[a]: Amp^r, apramycin resistance; Kan^r, kanamycin resistance; Amp^r, ampicillin resistance.

Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

2.3. Construction of S. paulus Δpau17

The *pau17* gene disruption mutant *S. paulus* Δ*pau17* was constructed by replacing the target gene with the kanamycin resistant gene cassette (*aph*). The 2.0-kb upstream fragment of *pau17* was amplified using primer pair *pau18*ES(AAAACTGCAGCATATGACCACCGACCAGGCCCG)/*pau18*ER(CGGGATCCGCCGCCTGCGATGACGAAC). The 1.5-kb downstream fragment of *pau17* was amplified using primer pair *pau16*ES (TAAAACGACGGCCAGTGAATTCCATATGGCGTCGCGCCACCGGCC)/*pau16*ER(TTCGGGTACCGAGCTCGAATTGCACGGGAGCCGGTCCTGAG). The two fragments were inserted into the *Pst*I/*Bam*HI and *Eco*RI sites of pUC119::KanR by ligation and ligation independent cloning (LIC) method [14], respectively, to generate an intermediate plasmid. The 4.5-kb mutant allele containing the up- and downstream-fragments of *pau17* and the kanamycin resistance cassette was excised from the intermediate plasmid by *Pst*I/*Eco*RI and inserted into the same sites of pKC1132 to afford plasmid pKC1132-*pau17*, which was then introduced into *S. paulus* NRRL 8115 *via E. coli-Streptomyces* conjugation. Exconjugants with kanamycin resistance were selected as the desired *Δpau17* mutants, and one of them confirmed by PCR was designated *S. paulus* Δ*pau17*.

2.4. Complementation of the S. paulus Δpau17 mutant

To complement *S. paulus* Δ*pau17*, a 1.2-kb DNA fragment containing the whole *pau17* gene was amplified from *S. paulus* NRRL 8115 genome using primer pair *pau17*ES(AAAACTGCAGCATATGGCGAAGTTCGTCATCGCAG)/*pau17*ER(CGGGATCCGTTCGCTGGTCACCTGCCTC) and inserted into the *Nde*I/*Bam*HI sites of pSET152::ermE* to afford the complementary plasmid pSET152-*pau17*. Introduction of pSET152-*pau17* into *S. paulus* Δ*pau17* by E. *coli-Streptomyces* conjugation generated the complementary strain *S. paulus* com-*pau17*.

2.5. Fermentation of the S. paulus strains

To check the production of PAUs of different *S. paulus* strains, 50 μL spores of *S. paulus* NRRL 8115 or mutants were inoculated into GS-7 liquid medium and cultured at 28 °C, 220 rpm for 2 days. Then the seed culture was transferred into 50 mL R5α liquid medium at a 2% ratio (v/v) and cultured for 4 days. The resulting broth was harvested by centrifugation, extracted with 50 mL ethyl acetate three times, and concentrated *in vacuo*. The samples were re-dissolved in 1 mL acetonitrile before HPLC analysis.

2.6. Expression and purification of Pau17

The 1.2-kb *pau17* fragment amplified from the *S. paulus* NRRL 8115 genome using primer pair *pau17*ES/*pau17*ER (with *Nde*I and *Bam*HI ends) was inserted into *Nde*I/*Bam*HI sites of pET28a to afford the *pau17* expression plasmid pET28a-*pau17*. A single transformant of *E. coli* BL21 (DE3)/pET28a-*pau17* was inoculated into LB (with 50 μg/mL kanamycin) and cultured overnight at 37 °C, 220 rpm. The overnight culture was used to inoculate LB medium (with 50 μg/mL kanamycin) at 1:100 dilution and incubated at 28 °C, 220 rpm until OD_{600} reached 0.6. Expression of Pau17 was then induced by the addition of isopropyl-β-thiogalactoside (IPTG) at a final concentration of 0.1 mM and cultured at 16 °C, 180 rpm for further 18–20 h.

Pau17 was purified using the Ni-NTA affinity column following the instruction of the manufacture (Novagen). At first, the cells were harvested by centrifugation, re-suspended in lysis buffer (20 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, and 5% glycerol, pH 7.9) and burst by ultrasonication. The cell debris was removed by centrifugation (16,000×*g*, 30 min). The supernatant was loaded onto the Ni-NTA affinity column pre-equilibrated with lysis buffera and washed with washing buffer (lysis buffer with 60 mM imidazole) and elution buffer (lysis buffer with 250 mM imidazole) stepwise. All steps were conducted under 4 °C. The purified protein was then desalted and concentrated by ultracentrifugation and stored at −80 °C in 50 mM PBS (pH 7.4) with 20% glycerol until use.

2.7. Enzymatic assays of Pau17

The assays of Pau17 were tested in a 50 μL mixture containing 50 mM PBS (pH 7.4), 2 mM NADH, 0.1 mM FAD, 1 mM 3-HAA (or mimic substrate 3-AHA, 3-HBA, or 2,3-DHBA), 5 μM Pau17 at 30 °C for 1 h. The reaction was quenched by equal volume of $CHCl_3$. After centrifugation, the aqueous phase of the reaction (20 μL) was subjected to HPLC analysis.

2.8. Preparation of 2,3,6-trihydroxybenzoic acid

2,3,6-trimethoxybenzoic acid (0.1 g, 0.47 mmol) was dissolved in 10 mL of dichloromethane in a flask at −80 °C. The flask is filled with N_2. A solution of (0.12 g, 0.71 mmol) of boron tribromide in 10 mL of dichloromethane was added to the stirred solution. The reaction mixture is allowed to warm up to room temperature overnight with stirring. After that, the reaction mixture was decanted to 10 mL of ice water to remove any excess of boron tribromide. The organic phase was separated, and the aqueous phase was extracted with ethyl acetate three times. The combined organic phases were dried over with anhydrous Na_2SO_4. Afterward, the solvents were removed under reduced pressure to give 2,3,6-trihydroxybenzoic acid (2,3,6-THBA, 0.03g, 40%), which was identified by HR-MS [15].

2.9. Spectroscopic analysis

HPLC analysis of PAUs was carried out on a Shimadzu HPLC system (Shimadzu, Kyoto, Japan). The analysis of paulomycin production was performed using an Apollo C18 column (5 μm, 4.6 × 250 mm, Alltech, Deerfield, IL, USA), which was developed with a linear gradient using acetonitrile and water with 0.1% trifluoroacetic acid at a flow rate of 0.8 mL/min. The ratio of acetonitrile was maintained at 5% for 5 min and changed linearly from 5% to 90% over 5–25 min and from 90% to 100% over 25–30 min. The detection wavelength was 320 nm.

HPLC analysis of the Pau17 assays was carried out with a ZORBAX SB-Aq StableBond Analytical column (5 μm, 4.6 × 250 mm, Agilent Teologies, Santa Clara, CA, USA). A linear gradient of acetonitrile and water with 0.1% trifluoroacetic acid was used for development of the column at a flow rate of 0.8 mL/min. The ratio of acetonitrile was changed linearly from 1% to 15% over 0–15 min, maintained at 15% for 3 min, from 15% to 50% over 18–28 min, from 50% to 100% over 28–30 min and maintained at 100% for 5 min. The detection wavelength was 320 nm.

LC-MS analysis was performed with the ZORBAX SB-Aq StableBond Analytical column (5 μm, 4.6 × 250 mm, Agilent Teologies, Santa Clara, CA, USA) on an Agilent 1260/6460 Triple-Quadrupole LC/MS system (Santa Clara, CA, USA). NMR spectra were recorded at room temperature on a Bruker-500 NMR spectrometer (Billerica, MA, USA).

3. Results

3.1. In vivo characterization of Pau17

In silico analysis revealed that Pau17 shares 44% identity with XlnD, a well-defined 3-hydroxybenzoate 6-hydroxylase involved in the degradation of 2,5-xylenol in *Pseudomonas alcaligenes* NCIMB 9867 [16]. Sequence alignment suggested that Pau17 belongs to group A flavin-dependent monooxygenase based on the facts that (*i*) Pau17 showed a high similarity to the group A prototype protein 4-hydroxybenzoate 3-hydroxylase from *Pseudomonas fluorescens* (UniProt ID: P00438, 30.4% similarity, 20% identity); (*ii*) all three fingerprint sequences of group A flavin-dependent monooxygenase could be observed in Pau17 including the GxGxxG motif and the GD motif for FAD binging and the DG motif serving a dual role of both FAD and NAD(P)H binding (Fig. 2A) [17–20].

To study the function of Pau17, *S. paulus* Δ*pau17* was constructed by replacing *pau17* with a kanamycin resistance gene cassette (Fig. 2B). The mutant strain was cultured at the PAU producing conditions and analyzed by HPLC. It was revealed that the production of PAUs was totally abolished in *S. paulus* Δ*pau17* (Fig. 2C). To exclude the possibility of a polar effect, this mutant was complemented by expressing *pau17 in trans*, which restored the synthesis of PAUs efficiently (Fig. 2C). These results showed that Pau17 is necessary for PAU production. Moreover, no PAU biosynthetic intermediate or shunt product was detected in *S. paulus* Δ*pau17*, indicating that Pau17 acts at the early stage of PAU biosynthesis.

3.2. In vitro characterization of Pau17

To further characterize Pau17, it was expressed as an *N*-His_6-tagged protein in *E. coli* BL21 and purified by Ni-NTA affinity chromatography (Fig. 3A). We assumed 3-HAA as a substrate of Pau17 based on the *in silico* and *in vivo* analyses of Pau17 and the fact that 3-HAA is an intermediate of PAU biosynthesis. To test this hypothesis, Pau17 was incubated with 3-HAA, FAD, and NADH at 30 °C, pH 7.4. HPLC analysis of the Pau17 assay showed that Pau17 can convert 3-HAA to a new product RA-318 along with the consumption of NADH (Fig. 3B). However, MS analysis of RA-318 showed that it is not the anticipated hydroxylized 3-HAA (169 Da) but a compound with much larger molecular weight (HR-MS *m/z* 319.0567 $[M+H]^+$) (Fig. 3C).

In order to determine the structure of RA-318, the Pau17 reaction was scaled up and 6.0 mg of RA-318 was prepared for NMR analysis, which suggested that RA-318 is a heterodimer of 3-HAA

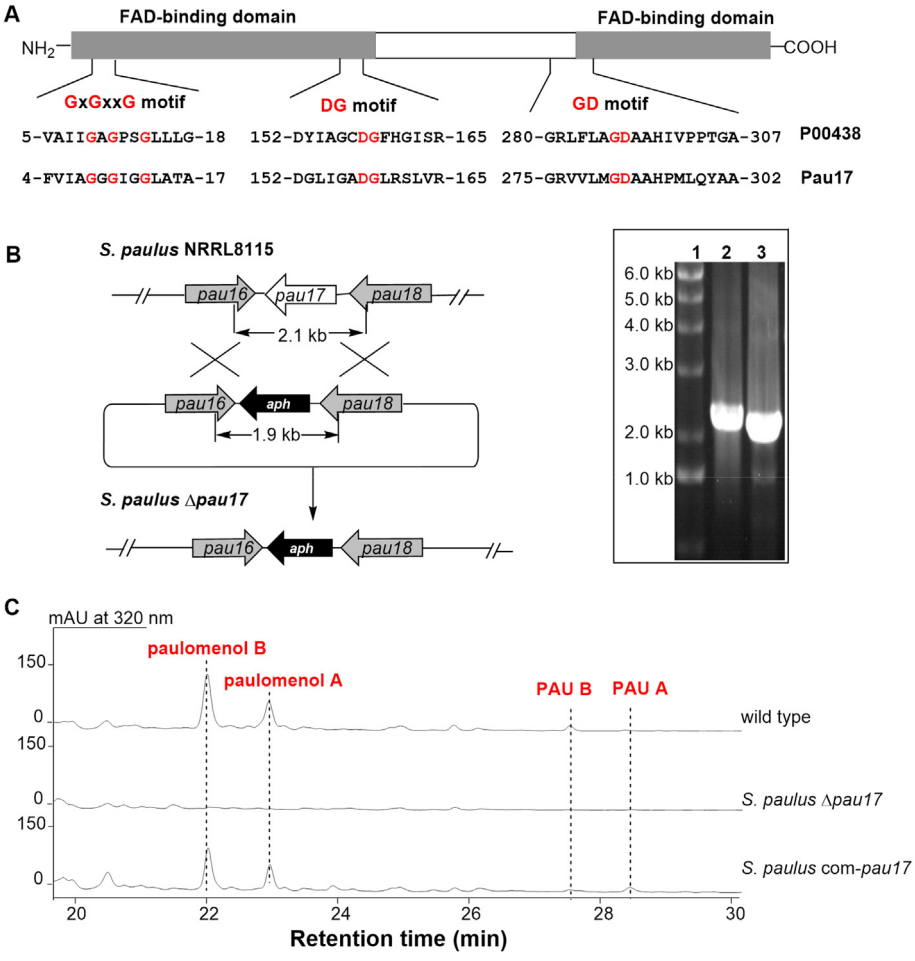
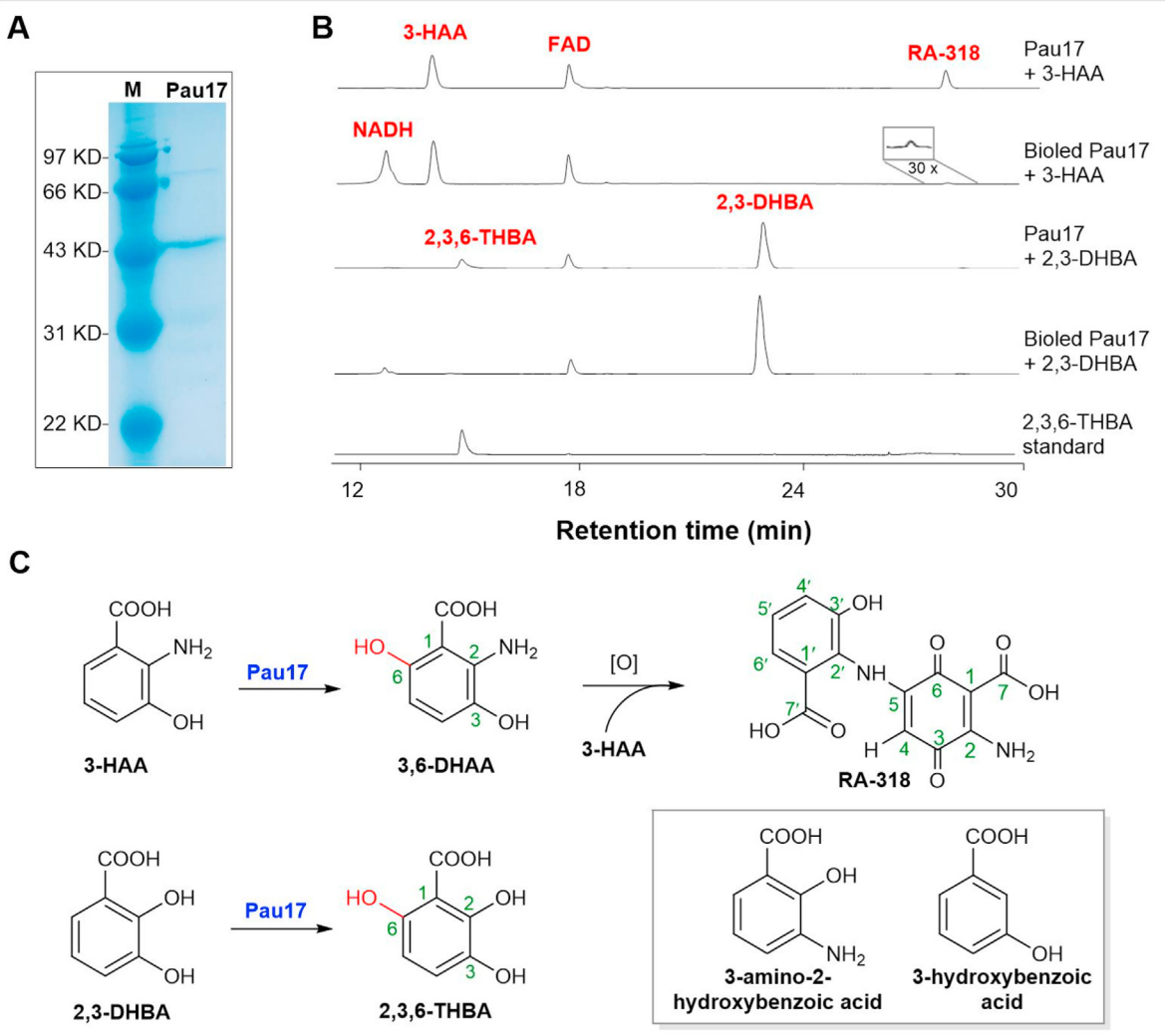


Fig. 2. *In silico* and *in vivo* characterization of Pau17. (A) Sequence comparison of Pau17 with 4-hydroxybenzoate 3-hydroxylase from *Pseudomonas fluorescens*. Upper panel: schematic representation of the primary structure of 4-hydroxybenzoate 3-hydroxylase from *P. fluorescens* (UniProt ID: P00438) with the FAD-binding domain in light gray and the substrate binding domain in white. The positions of the three fingerprint sequences of group A flavin-dependent monooxygenase were also showed. Lower panel: sequence alignment of Pau17 and P00438 at the fingerprint sequence regions. (B) Construction of *S. paulus* Δ*pau17* (left panel) and genotype verification of the mutant by PCR (right panel). Lane 1, DNA marker; lane 2, *S. paulus* NRRL 8115; lane 3, *S. paulus* Δ*pau17*. The sizes and positions of the PCR fragments amplified by PCR for genotype verification were showed on the left panel. (C) HPLC metabolic profiles of the *S. paulus* Δ*pau17* gene inactivated mutants *S. paulus* Δ*pau17* and the complemented strain *S. paulus* com-*pau17*.



D

^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data of RA-318 in DMSO-d_6

No.	δ_H (ppm, *J*=Hz)	δ_C (ppm)	No.	δ_H (ppm, *J*=Hz)	δ_C (ppm)
1		94.87	1′		123.28
2		157.80	2′		129.45
3		180.28	3′		152.18
4	5.05 (s, 1H)	99.12	4′	7.18 (d, 8.0,1H)	120.78
5		148.58	5′	7.28 (t, 7.6 and 8.0, 1H)	128.38
6		174.71	6′	7.41 (d, 7.6, 1H)	121.98
7		168.41	7′		167.95

Fig. 3. *In vitro* characterization of Pau17 as a 3-HAA 6-hydroxylase. (A) SDS-PAGE analysis of Pau17. M, protein marker; Pau17, *N*-His$_6$-tagged Pau17. (B) HPLC analysis of representative enzymatic assays of Pau17 using 3-HAA or 2,3-DHBA as a substrate. (C) Reactions catalyzed by Pau17 in the enzymatic assays. Two 3-HAA analogues that cannot be taken by Pau17 were also presented in the gray square. (D) ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data of RA-318 in DMSO-d_6.

and 3,6-diketo-anthranilic acid (Fig. 3D)). Previously, RA-318 was reported as a product of 3-HAA slow auto-oxidation under alkaline conditions (pH 11.7) exposing to air [21]. In accordance with this, a tiny amount of RA-318 could be detected in the control assay using boiled Pau17 (Fig. 3B). These results implied that Pau17 can catalyze the 6-hydroxylation of 3-HAA to generate 3,6-dihydroxyanthranilic acid (3,6-DHAA), which is then converted to RA-318 by spontaneous oxidation and a nucleophilic coupling reaction (Fig. 3C).

3.3. Pau17 can catalyze the 6-hydroxylation of 2,3-DHBA

The substrate promiscuity of Pau17 was then tested using 3-amino-2-hydroxybenzoic acid, 3-hydroxybenzoic acid, and 2,3-DHBA to substitute 3-HAA. Although the former two could not be recognized by Pau17, the last candidate, 2,3-DHBA, was oxidized by Pau17 to generate 2,3,6-trihydroxy benzoic acid (2,3,6-THBA), which was identified by HR-MS analysis (*m/z* 169.0138 $[M+H]^+$, calcd 169.0142) and a HPLC co-injection with authentic 2,3,6-THBA (Fig. 3B). The 2,3-DHBA 6-hydroyxlation activity of Pau17 showed that this enzyme has certain substrate promiscuity towards 3-HAA analogues. In addition, the conversion of 2,3-DHBA to 2,3,6-THB provided further evidence to support that Pau17 acts as a 3-HAA 6-hydroxylase in PAU biosynthesis.

4. Discussion

As a group of antibiotics with considerable inhibition activity against tumor cell lines and varied gram-positive bacteria discovered in 1960s (named as proceomycin or U-43,120 originally) [3,22], PAUs were intensively studied for their potential use in the treatment of infection diseases [23,24]. PAUs exhibited attracting activities against some gram-positive bacteria pathogens including multiple-antibiotic-resistant *Staphylococcus aureus* [23]. They could also kill the *S. aureus* surviving within human or bovine polymorphonuclear leukocytes efficiently [24]. The biosynthetic studies of PAUs were initiated by the feeding experiments of various precursors, which suggested that the fatty acyl chains connected to paulomycose 7″-OH of PAU A and B are derived from the degradation products of isoleucine and valine, respectively [25]. Some important progresses on understanding PAU biosynthesis have been made after the *pau* biosynthetic gene clusters were assigned. In the case of the special ring A moiety of PAUs, it was proposed that Pau18 (Plm15), Pau19 (Plm16), and Pau20 (Plm17) can convert chorismate to 3-HAA, an identified intermediate of PAU biosynthesis [4,7,9]. We showed that Pau17 could convert 3-HAA and 2,3-DHBA to RA-318 and 2,3,6-THBA, respectively. Both of the two enzymatic assays and the *in vivo* results supported that Pau17 is a 3-HAA 6-hydroxylase involved in PAU biosynthesis. In the PAUs producers, the product of Pau17, 3,6-DHAA, may be taken by the following biosynthetic enzymes immediately to avoid the attacks from cellular nucleophiles and auto-oxidation.

One obstacle for the drug development of PAUs is their instability, which is caused by the spontaneous detachment of paulic acid moiety to generate paulomenols (under alkaline conditions) or the dehydration between C-4 and C-5 to form a quinone-type ring A (under acidic conditions) [6]. To stabilize the paulic acid part, PAUs were derived to paldimycins by adding *N*-acetyl-L-cysteine to the fermentation media of *S. paulus* strains or by semi-synthetically mixing PAUs with *N*-acetyl-L-cysteine in aqueous condition (pH 8.6) at room temperature [6,26]. Paldimycins, with the isothiocyanate containing paulic acid protected by addition reactions with *N*-acetyl-L-cysteine, are much stable than PAUs at acidic or alkaline conditions. Unfortunately, the minimum inhibitory concentrations (MICs) of paldimycins against the tested gram-positive bacteria pathogens are about one magnitude higher than those of PAUs [6]. Recently, we substituted *N*-acetyl-L-cysteine with *N*-acetylcysteamine and generated palsimycins A and B, which retained the antibacterial activities of PAUs and were much stable under both acidic and alkaline conditions, severing a practical way to stabilize the paulic acid moiety of PAUs [27]. If the biosynthetic mechanism of ring A is well understood, it may inspire combinatorial biosynthesis to generate modified PAUs with better activities and/or stabilities. Here, we push one step forward toward elucidating the biosynthetic mechanism of PAU ring A by characterizing Pau17 as an unprecedented 3-HAA 6-hydroxylase. The product of Pau17 catalyzed reaction, 3,6-DHAA, may undergo further C-6 glycosylated and C-4 hyoxylation to shape the special ring A structure of PAUs. Those biosynthetic steps are currently being actively investigated in our laboratory.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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