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A key cytochrome P450 hydroxylase in pradimicin biosynthesis

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Abstract

Pradimicins A–C (**1–3**) are a group of antifungal and antiviral polyketides from *Actinomadura hibisca*. The sugar moieties in pradimicins are required for their biological activities. Consequently, the 5-OH that is used for glycosylation plays a critical role in pradimicin biosynthesis. A cytochrome P450 monooxygenase gene, *pdmJ*, was amplified from the genomic DNA of *A. hibisca* and expressed in *Escherichia coli* BL21(DE3). PdmJ introduced a hydroxyl group to G-2A (**4**), a key pradimicin biosynthetic intermediate, at C-5 to form JX134 (**5**). A D-Ala-containing pradimicin analog, JX137a (**6**) was tested as an alternative substrate, but no product was detected by LC-MS, indicating that PdmJ has strict substrate specificity. Kinetic studies revealed a typical substrate inhibition of PdmJ activity. The optimal substrate concentration for the highest velocity is 115 μ M under the test conditions. Moreover, the conversion rate of **4** to **5** was reduced by the presence of **6**, likely due to competitive inhibition. Coexpression of PdmJ and a glucose 1-dehydrogenase in *E. coli* BL21(DE3) provides an efficient method to produce the important intermediate **5** from **4**.

Keywords

Pradimicins; Cytochrome P450 monooxygenase; Hydroxylation; Substrate inhibition; *Actinomadura hibisca*

Pradimicins A (**1**), B (**2**), and C (**3**) (Fig. 1A) are a group of benzo[α]naphthacenequinone natural products isolated from *Actinomadura hibisca* P157-2 as antifungal antibiotics.¹ Other analogs such as pradimicins D and E have also been isolated from actinomycetes.^{2–6} The structure of **1** contains an amino acid D-Ala, two sugar moieties including D-xylose and 4,6-dideoxy-4-methylamino-D-galactose, and a dihydrobenzo[α]naphthacenequinone aglycon. This unique structure makes **1** an effective viral entry inhibitor,^{7,8} and a broad-spectrum fungicide.⁹ The sugar moieties attached to C-5 are found essential for the biological activities of **1**. Consequently, C-5 hydroxylation is a key step in pradimicin biosynthesis by providing the 5-OH for the introduction of the 4,6-dideoxy-4-methylamino-D-galactose moiety. Identification of the dedicated biosynthetic enzyme thus is important for understanding the biosynthetic pathway of **1**.

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Supplementary Data

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Pradimicins belong to a class of natural products called polyketides. Similar to fatty acid biosynthesis, polyketides are assembled through stepwise condensations of simple carboxylic acid precursors catalyzed by enzymes known as polyketide synthases (PKSs).¹⁰ Three types of PKSs have been identified and designated as types I, II and III. Pradimicins are synthesized through a type II PKS pathway and the pradimicin biosynthetic gene cluster from *A. hibisca* P157-2 has been reported.¹¹ We have previously investigated the early biosynthetic steps that are involved in the formation of the pentangular polyphenol structure. The minimum set of enzymes required to afford the benzo[*a*]naphthacenequinone core structure includes PdmA, B, C, D, H, K and L. PdmA, B and C act as ketosynthase, chain length factor and acyl carrier protein, respectively, which form the minimal PKS. PdmD, K and L are three dedicated cyclases, while PdmH is a monooxygenase that synergistically works with the cyclases to form the pentangular structure. Addition of a ketoreductase, PdmG, led to the biosynthesis of a key intermediate G-2A (**4**) (Fig. 1B).¹² This intermediate is subjected to a series of modifications by other tailoring enzymes to afford the final structure of **1**.

PdmJ and PdmW are two putative cytochrome P450 (CYP) monooxygenases found in the pradimicin biosynthetic gene cluster. CYP enzymes are widely distributed in various organisms. They play important physiological roles in the oxidative metabolism of endogenous and exogenous compounds, and are also frequently found in natural product biosynthetic pathways. Through a combinatorial biosynthesis approach, PdmJ and PdmW were characterized as a C-5 hydroxylase and a C-6 hydroxylase, respectively. Coexpression of PdmJ with PdmABCDGHKL in *S. coelicolor* CH999 gave rise to the C-5 hydroxylated product of **4**, JX134 (**5**, Fig. 1B). Similarly, a C-6 hydroxylated derivative of **4**, JX152, was generated when PdmW was introduced. However, the yields of both hydroxylated products were low,¹³ which has hampered further work on other downstream tailoring enzymes. Moreover, the lack of *in vitro* studies has hampered the understanding of these key biosynthetic enzymes. In this study, we report the reconstitution of PdmJ in *Escherichia coli* BL21(DE3) and biochemical characterization of this enzyme as the C-5 hydroxylase. The recombinant enzyme can efficiently convert **4** into **5** both *in vitro* and *in vivo*.

The deduced protein product of *pdmJ* has 411 amino acids. BLAST analysis of the sequence revealed numerous CYP monooxygenases, among which PdmJ shows the highest homology to LlpOVI with 57% identity and 71% similarity. The sequence alignment of PdmJ with LlpOVI, PdmW and PikC is shown in Fig. 2. LlpOVI is a putative CYP monooxygenase that is involved in lysolipin biosynthesis in *S. tendae* Tü 4042.¹⁴ PikC is a CYP hydroxylase from the methymycin/pikromycin biosynthetic pathway in *S. venezuelae*,¹⁵ while PdmW is a CYP hydroxylase of the pradimicin biosynthetic pathway that is responsible for C-6 hydroxylation.¹³ As shown in Fig. 2, several typical conserved motifs in CYP enzymes were identified (boxed) in PdmJ. Motif 1 G-X-[DEH]-T represents an oxygen-binding site and point of access for an incoming dioxygen molecule.^{16, 17} Motif 2 E-X-X-R, which is conserved in all CYP enzymes, is also present in PdmJ. This motif is proposed to participate in both the redox partner interaction and heme binding.¹⁷ Motif 3 represents the CYP Cys heme-iron ligand signature [FW]-[SGNH]-X-[GD]-{F}-[RKHPT]-{P}-C-[LIVMFAP]-[GAD] as indicated in the PROSITE database (Accession number PS00086).¹⁸ Altogether these features strongly suggest that PdmJ is a CYP monooxygenase.

The gene *pdmJ* was cloned from the genomic DNA of the pradimicin producing strain and ligated into pJET1.2 to yield pZJ41. After confirming the sequence of the PCR product, *pdmJ* was ligated into pACYCDuet-1 to yield the expression plasmid pKN36 (Supplementary Fig. 1). The plasmid was transformed into *E. coli* BL21(DE3) and expressed at 28°C. SDS-PAGE analysis of the soluble and insoluble fractions of the cell lysate indicated that both fractions contained PdmJ. The protein was then purified from the

soluble fraction by Ni-NTA chromatography to homogeneity. PdmJ was found in the eluent using buffer A (50 mM Tris-HCl, pH 7.9, 2 mM EDTA, 2 mM DTT and 0.1 μ M PMSF) containing 25 mM imidazole (Supplementary Fig. 2). SDS-PAGE analysis showed that the N-terminal His₆-tagged enzyme was pure and had the correct size (47 kDa). The yield of PdmJ was estimated to be 17.8 mg/L using a bicinchoninic acid protein assay. The purified PdmJ was subjected to spectroscopic analyses. An absolute spectrum of 5 μ M ferric PdmJ in 50 mM Tris-HCl buffer (pH 7.9) with 20% glycerol generated at room temperature showed Soret, α and β bands at 417, 570 and 535 nm, respectively. Second-derivative analysis of the Soret peak indicated that about 90% of the iron was in the low-spin state (Fig. 3A). The CO-difference spectra¹⁹ of PdmJ (Fig. 3B) showed a peak at 450 nm immediately after the addition of 2.5 mM dithionite to the enzyme saturated with CO, indicating that PdmJ is a CYP enzyme. However, the P450 form is unstable and rapidly changes to the P420 form, as revealed by the formation of the peak at 420 nm which increased after each reading (1-min intervals) while the peak at 450 nm diminished.

With the purified PdmJ in hand, we next tested its activity through *in vitro* reactions. In a previous *in vivo* study, heterologous expression of PdmJ with the enzymes (PdmABCDGHLK) that form **4** in *S. coelicolor* CH999 yielded the hydroxylated product **5**.¹³ This indicated that **4** is a substrate of PdmJ. Accordingly, we isolated **4** from *S. coelicolor* CH999/pJX120 (Supplementary Figs. 3 and 4) and used it as the substrate in our *in vitro* reactions. The 100 μ L reaction mixture contained 100 mM Tris-HCl buffer (pH 7.9), 10 μ g of spinach ferredoxin, 0.05 U spinach ferredoxin-NADP reductase, 1.5 mM NADPH, 133 μ g of PdmJ, and 200 μ M of **4**. The reaction was started by adding NADPH at 28°C and quenched after 10 min with 50 μ L of methanol. The reaction was analyzed on an Agilent 1200 LC/MS with a C₁₈ Eclipse Plus column (5 μ m, 4.6 $\times$ 250 mm), eluted with a linear gradient (10 to 90% acetonitrile in water containing 0.1% trifluoroacetic acid over 25 min). LC-MS analysis of the reaction mixture showed that PdmJ had converted **4** into a more polar product (ca. 47.8% yield) at 19.6 min (trace ii, Fig. 4A). This product was identified as **5** by comparing the UV spectrum (Supplementary Fig. 5), molecular weight (448 Da, Supplementary Fig. 6), and retention time with those of an authentic sample, clearly confirming that PdmJ is the C-5 hydroxylase, which is in agreement with the *in vivo* result previously reported.¹³

To study the kinetics of PdmJ, a wide range of concentrations of **4** (32–920 μ M) were tested in the reactions. As shown in Fig. 3C, when the substrate was present at low concentrations, the reaction rate increased with the concentration. The reaction rate reached the highest level, 0.7 nmol/min, when the concentration of **4** was 115 μ M. When more substrate was provided, the reaction rate declined. A non-Michaelis-Menten kinetics curve obtained from the experimental data (Fig. 3C) is likely due to substrate inhibition at high concentrations. It is known that **1–3** are the major secondary metabolites of *A. hibisca* P157-2. The isolation yields of **1–3** in the production medium were 290.9, 26.8 and 18.8 mg/L, respectively.¹ It is proposed that the enzymes in the pradimicin biosynthetic pathway are tightly controlled so that the intermediates can be efficiently utilized by the immediate downstream enzymes. Consequently, the physiological concentration of the biosynthetic intermediates including **4** in *A. hibisca* P157-2 is low and substrate inhibition of PdmJ in this pradimicin producing strain does not occur. In fact, it is normal in metabolism that the concentrations of intracellular intermediates are much lower than the metabolic fluxes. This explains the high yields of **1–3** in *A. hibisca* P157-2 despite the substrate inhibition of PdmJ.

To examine the substrate specificity of PdmJ, JX137a (**6**) (Supplementary Figs. 7 and 8), a D-Ala containing analog of **4**, was isolated from *S. coelicolor* CH999/pJX137 and used for the hydroxylation assay. **6** is identical to **4** except for the D-Ala moiety. No formation of a hydroxylated product was detected (trace iv, Fig. 4A), which indicated that PdmJ has strict

substrate specificity. Our previous study has shown that **6** is the product of coexpression of PdmN with PdmABCDGHL in *S. coelicolor* CH999.¹³ Since **6** appears not to be a substrate for PdmJ, it is likely that PdmN has flexible substrate specificity and the amino acid ligation catalyzed by PdmN might be a tailoring step that occurs after C-5 hydroxylation. Given their similar structures, we also tested whether **6** can interfere with the hydroxylation of **4** by competing for the substrate binding site of PdmJ. In the presence of **6** at 200 μ M and 400 μ M, the conversion rates of **4** to **5** were reduced by about 40% and 80%, respectively. This indicated that although **6** is not a substrate of PdmJ, it can still bind to the enzyme as a competitive inhibitor.

Although lacking the flavoprotein NADPH-CYP reductase that ubiquitously exists in eukaryotic cells, *E. coli* is capable of supporting the activities of heterologous CYPs.²⁰ We next attempted to feed **4** to *E. coli* BL21(DE3)/pKN36 for its bioconversion to **5**. The substrate was added to growing cultures 3 h after IPTG induction and growth was continued at 28°C for an additional 30 h. The culture was centrifuged and LC-MS analysis of the methanol extract of the sedimented cells showed that **4** was converted into **5** (ca. 7.3% yield) by the engineered strain (trace ii, Fig. 4B). No bioconversion was observed by the plasmid control strain *E. coli* BL21(DE3)/pACYCDuet-1 that lacks *pdmJ* (trace i, Fig. 4B).

Glucose 1-dehydrogenase (GDH) oxidizes D-glucose into D-glucono- δ -lactone in the presence of cofactor NAD⁺ or NADP⁺ to regenerate NAD(P)H. Coexpression of GDH with CYP enzymes significantly improves the *in vivo* efficiency of several CYP enzymes.^{21, 22} We thus tested whether coexpression of GDH and PdmJ would improve the rate of bioconversion of **4** to **5**. Both *gdh* (from *Bacillus subtilis*) and *pdmJ* were cloned into pACYCDuet-1 to yield pKN29 that harbors the genes in two different multiple cloning sites and under the control of separate T7 promoters (Supplementary Fig. 1). As shown in Fig. 4B, coexpression (trace iii) resulted in a higher bioconversion rate (ca. 67.4% yield) in the presence of 5 g/L glucose compared to that of *E. coli* BL21(DE3)/pKN36, which is likely attributed to the efficient regeneration of NADPH. The yield may be further improved by coexpressing a NADPH-CYP reductase with PdmJ and GDH.

Pradmicins represent a novel family of medicinally important natural products because of their significant antifungal and antiviral activities. Deciphering the biosynthetic mechanisms of these pentangular polyphenols will advance the overall understanding of polyketide biosynthesis and provide a basis for engineering the synthesis of “unnatural” natural products for new drug discovery. We have previously investigated the early biosynthetic steps using an *in vivo* combinatorial biosynthesis approach. Given the importance of the 5-OH in pradimicin biosynthesis and the inefficient synthesis of **5** in *Streptomyces*, we reconstituted PdmJ in *E. coli* BL21(DE3) in this study and purified the enzyme for biochemical characterization. Our *in vitro* studies revealed that PdmJ can take **4** as the substrate and efficiently introduce a hydroxyl group to the molecule at C-5 to form **5**. This hydroxyl group serves as a site for subsequent glycosylations. The enzyme has narrow substrate specificity, as revealed by the fact that it failed to hydroxylate the highly similar compound **6**. Interestingly, the activity of this enzyme can be inhibited by **4** (substrate inhibition) and **6** (competitive inhibition). Future structural analysis of the enzyme may reveal valuable information about the substrate binding and catalytic sites of PdmJ. Although substrate inhibition of CYP enzymes has been previously studied in drug metabolism,^{23, 24} this is the first time that a CYP hydroxylase in a type II polyketide biosynthetic pathway was found to be inhibited by its substrate.

In vivo bioconversion of **4** to **5** was also achieved at a high yield by feeding the substrate to the induced fermentation broth of the *E. coli* strain that expresses PdmJ and GDH. Additionally, it should be noted that the bioconversion of **4** to **5** can be done in 2 d, rather

than 8 d that is required for *S. coelicolor* CH999/pJX134 to produce **5**.¹³ Thus, bioconversion by *E. coli* BL21(DE3)/pKN29 is useful for producing **5** as an important pradimicin biosynthetic intermediate, which can be used as a starting molecule for further chemical or enzymatic glycosylations to generate novel pradimicin analogs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Structures and the biosynthetic pathway of pradimicins. (A) Structures of pradimicins A (**1**), B (**2**), C (**3**) and JX137a (**6**). (B) Biosynthetic pathway of **1**.

PdmJ 1 MPSSKDAPTVDPRPDVTPAFFFRPDDPFQPPCEHARLRASDFVAKVVLPTGDHAWVTRYADVRFVTSRRRFSKEAVTRPGAPRLIPMQ 89
 LlpOVI 1 MSTEDGVRHFFFAASDPVEPPDECAALLREEFVARVRIPPTGDQVWLVRHEDVRRVLSDFRFSREAITAPGAPRLPIA 79
 PdmW 1 METTLPYPFRRPSALEPPAELRGLR-EQPVVKVVLPSGDEAWMTRYADVRALLADDRISRN-LNRFGAARISAKN 75
 PikC 1 MRRTQQGTTASPPVLDLALGQDFAADPYPTYARLRAGEPAHRVRTPEGDEVWLVVGYDRARAVLADPRFSKD-WRNSTTPLTEAEA 86

PdmJ 90 RGSKS-LVIMDPPEHTRMRKIVSRAFTARRVEGMRRAHVRLDTSGLFVDEMVEHGPP-ADLIAHLALPLFVTVICEMLGVPPEDRPRE 171
 LlpOVI 80 EGSKS-IFVMDPPEHSRLRLVSRFSPRRIESLRDVRQLAEDLVDAMVDQGPP-ADLIAGLAQPLPITVICRMLGVPEYDERVF 163
 PdmW 76 KLFQDPKMDPPPEHTRIRRLVMKALTPSRVERLRPRVQEITDRLVTAMAERRPP-IDVNEALAYPLPIQVVCCELLGVPEYDERVF 160
 PikC 87 ALNHN-MLESDDPRHTRLRKLVAREFTMRRVELLRPRVQEIVDGLVDAMLAPDGRADLMESLAWPLPITVISELLGVPEPDRAAF 171

PdmJ 172 QDWTDRMLTIGAPALAQADEIKAAVGRGLRGYLAELIDAKTAAPADDLLSLLSRAHAD-EG--LSEELLTFGMTLLAASYHTTTAA 256
 LlpOVI 164 REWTDVMLS YARDGREQ---VAAARDSLSGYLTELIDAKRAHPTDDLMLVLTAAADDGDR--LDDAELLAFGYTLLGAGYHATTAG 244
 PdmW 161 RVNTGAFLSISGYTQDE---IERNMSRLNEYMRDLIEAKRADPADDLISAMIAVTDAEDGR-LSEYELHWSRLVLVGFETTAVQ 242
 PikC 172 RVNTDAFVFPDDPAQAQ-----TAMAMSGYLSRLIDSKRGQDGEDLLSALVRSDE-DGSRLTSEELLGMAHILLVAGHETTAVNL 251

PdmJ 257 ITHSVYHLLEPSRYARLRDPSGIPAAVEELLRYGQIGGGAGAIRIAVEDVEVGGLVVRAGEAVIPLFNAANRDPEVFADPEELD 342
 LlpOVI 245 LVHALLALVRRPADLAALNRPELMPATAVDELLRRSQAGSGLGAMRIAEDEVIEHGVRIAAGDAVLFWINAAANRDPRVFDPPDGLL 330
 PdmW 243 IGGCIAMLLSRPDQLDLVRKDPALLRPAAVEELLRWKIVGSSVTMLRYVTEDVEVGVTIPAGSSIIIPAGDSANQDESVEHPEVFD 328
 PikC 252 IANGMYALLSHPDQLAALRADMTLLDGAVEEMLRV-EGPVESATYRFPVEFVDLDGTVIPAGDTVLVLADAHRTPERFPPDHRFD 336

PdmJ 343 LGRTDNPHIALGHGHIHYCLGAPLARLELQVLETIVERTPALRLAIDDADITWRPGLAFARPDALPIAW 411
 LlpOVI 331 LDRKPNALHSFGHGIHCLGAQLGRMELEIALGALLRRMPGLRLAVPEADLQWSSQLAFSRPRTLPAW 399
 PdmW 329 VTRTDNPHLTFSHGTHYCAGASLARLELVVLTSLIRRLPDRLAIPATDLRRNDGNLLESFREPITWG-GRG 401
 PikC 337 IRRDTAGHLAFGHGHIHFCIGAPLARLEARIAVRALLERCPDLALDVSPGELVWYPNPMIRGLKALPIRWRRGREAGRRTG 416

Figure 2.

Amino acid sequence alignment of PdmJ with three other actinomycete CYP monooxygenases. The identical amino acid residues are shadowed in grey. Three conserved regions in CYP monooxygenases are boxed.

Figure 3. Spectroscopic and enzymatic analyses of PdmJ. (A) Absolute spectrum of ferric PdmJ. (B) CO-difference spectra of PdmJ. (C) Substrate inhibition of PdmJ-catalyzed C-5 hydroxylation of **4**.

Figure 4.

HPLC analysis of C-5 hydroxylation by PdmJ at 460 nm. (A) *In vitro* reaction of PdmJ with **4** and **6**. (i) Standard sample of **4**. (ii) The reaction mixture of PdmJ with **4**. (iii) Standard sample of **6**. (iv) The reaction mixture of PdmJ with **6**. (B) *In vivo* bioconversion of **4** to **5** by engineered *E. coli*. (i) *E. coli* BL21(DE3)/pACYCDuet-1; (ii) *E. coli* BL21(DE3)/pKN36; (iii) *E. coli* BL21(DE3)/pKN29 with 5 g/L glucose.