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Three enzymes involved in the *N*-methylation and incorporation of the pradimicin sugar moieties

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Abstract

Pradimicins are antifungal and antiviral natural products from *Actinomadura hibisca* P157-2. The sugar moieties play a critical role in the biological activities of these compounds. There are two glycosyltransferase genes in the pradimicin biosynthetic gene cluster, *pdmS* and *pdmQ*, which are putatively responsible for the introduction of the sugar moieties during pradimicin biosynthesis. In this study, we disrupted these two genes using a double crossover approach. Disruption of *pdmS* led to the production of pradimicinone I, the aglycon of pradimicin A, which confirmed that PdmS is the O-glycosyltransferase responsible for the first glycosylation step and attaching the 4',6'-dideoxy-4'-amino-D-galactose or 4',6'-dideoxy-4'-methylamino-D-galactose moiety to the 5-OH. Disruption of *pdmQ* resulted in the production of pradimicin B, indicating that this enzyme is the second glycosyltransferase that introduces the D-xylose moiety to the 3'-OH of the first sugar moiety. Insertion of an integrative plasmid before *pdmO* might have interfered with the dedicated promoter, yielding a mutant that produces pradimicin C as the major metabolite, which suggested that PdmO is the enzyme that specifically methylates the 4'-NH₂ of the 4',6'-dideoxy-4'-amino-D-galactose moiety. Functional characterization of these sugar-decorating and -incorporating enzymes thus facilitates the understanding of the pradimicin biosynthetic pathway.

Keywords

Pradimicin A; Glycosyltransferase; Methyltransferase; Gene disruption; Polyketide

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Supplementary Material

Supplementary material that may be helpful in the review process should be prepared and provided as a separate electronic file. That file can then be transformed into PDF format and submitted along with the manuscript and graphic files to the appropriate editorial office.

Pradimicins A-C (**1-3**, Figure 1) are aromatic polyketide natural products from the soil bacterium *Actinomadura hibisica* P157-2. Since their discovery in 1988, these molecules have been intensively studied. **1** is a promising lead compound due to its combined antifungal/antiviral properties. It was found to be active against a broad-spectrum of opportunistic and pathogenic fungi. This compound also interferes with the recognition of HIV-1 to its target cells. The mechanism of action of **1** emphasizes its lectin-like property in the presence of Ca^{2+} .¹ The moieties of **1** form a primary cavity with C-14 and C-15 from the benzo[α]naphthacenequinone and several hydroxyl groups of D-mannopyranoside.² Based on the intermolecular distance in the proposed model, it is believed that the free carboxyl group at C-18 of two molecules of **1** interacts with one Ca^{2+} ion.² Another study on the anticandidal mode of action was done with the semisynthetic pradimicin derivative, BMY-28864.³ It was concluded that the sugar moieties of pradimicins, especially thomosamine or 4',6'-dideoxy-4'-methylamino-D-galactose, were critical for sugar-recognition and involved in binding to the specific mannan.

The pradimicin (*pdm*) biosynthetic gene cluster has previously been reported (NCBI accession number: EF151801.1). This reported gene cluster consists of 28 open reading frames.¹ Through combinatorial biosynthesis and heterologous expression, we have identified the enzymes that synthesize the pentangular intermediate G-2A, including PdmA, B, C, D, G, H, K, and L.² Two cytochrome P450 enzymes, PdmJ and PdmW, were functionally characterized as the dedicated hydroxylases that specifically introduce a hydroxyl group to C-5 and C-6, respectively.³ We have also identified PdmN as an amino acid ligase that induces a D-alanine moiety at C-16. This enzyme was found to possess broad substrate and donor specificity.^{3, 4} Synergistic actions of tailoring enzymes in pradimicin biosynthesis commonly exist to ensure an efficient assembly process. In this work, we show the functional characterization of three late tailoring enzymes, including PdmS, PdmQ and PdmO, using a homologous gene recombination approach. PdmS and PdmQ were characterized as the glycosyltransferases (GTs) that successively introduce the 4',6'-dideoxy-4'-methylamino-Dgalactose/ 4',6'-dideoxy-4'-amino-D-galactose and D-xylose moieties to the structure, while PdmO is responsible for the *N*-methylation of 4',6'-dideoxy-4'-amino-D-galactose at 4'-NH₂.

As in the pradimicin family, many other important antibiotics such as erythromycin naturally contain deoxysugar moieties attached to the aglycon. Because these sugar moieties often play a critical role in the bioactivities of these natural products, it is of great interest to identify the GTs responsible for transferring these deoxysugars to the aglycons. The *pdm* biosynthetic gene cluster contains two putative GT genes, *pdmS* and *pdmQ*. Sequence analysis indicated that they belong to a family of GTs that catalyzes the final stage of the biosynthesis of glycosylated natural products such as vancomycin and chloroeremomycin.

BLAST analysis revealed that PdmS (GenBank accession number ABK58688) is a putative GT that contains 437 amino acids (aa). Two proteins with the highest homology include two proposed GTs from *Paenibacillus dendritiformis* (GenBank accession number WP_006678995, 423 aa, 61% identity) and *Bacillus cereus* (GenBank accession number WP_002061747, 416 aa, 59% identity). However, none of these are functionally characterized. To understand the role of PdmS in pradimicin biosynthesis, we designed two

sets of primers to inactivate this gene using a double crossover approach. A 1482-bp left arm and a 1404-bp right arm were cloned from the genome of *A. hibisca* P157-2 (Fig. 2A). These two fragments were ligated to the thermal sensitive plasmid pKC1139 between HindIII and XbaI as well as XbaI and EcoRI, respectively, to yield a disruption plasmid pKN82. The primers used and plasmids constructed in this work are shown in Tables S1 and S2, respectively. The intergeneric conjugation between *E. coli* and *A. hibisca* has been described by Kiser et al.⁵ and pKN82 was introduced into *A. hibisca* P157-2 through a similar way.⁶ Correct transformants of *A. hibisca*/pKN82 were cultivated in YM broth (4 g L⁻¹ Dglucose, 4 g L⁻¹ yeast extract, and 10 g L⁻¹ malt extract) in the presence of 50 mg L⁻¹ apramycin at 28°C in a rotary shaker, and then cultivated on ISP4 plates with 50 mg L⁻¹ apramycin at 37°C for a period of 10 days to allow the plasmid to integrate into the genome. Conjugants were picked to YM broth without any antibiotics and incubated for 10 days at 28°C. The cultures were then spread onto ISP4 plates without antibiotics and growth for about 10 days at 28°C. The selection process for positive double crossover mutants was performed using a replica plate technique for colonies sensitive to 50 mg L⁻¹ apramycin. Positive colonies were cultivated in YM broth in a rotary shaker at 28°C and were subjected to PCR screening using a set of *pdmS*-specific primers. As shown in Figure 2B (panel i), a 1.3-kb fragment of *pdmS* can be amplified from the genome of the wild type, while a disrupted gene *pdmS** with a length of 0.9 kb was cloned from the correct double crossover mutant. This confirmed that a 0.4-kb fragment of *pdmS* has been successfully deleted from the genome of *A. hibisca* P157-2.

The *PdmS* mutant of *A. hibisca* P157-2 was then grown in YM medium for product analysis. The fermentation broth was centrifuged to separate the cells and supernatant, and the latter was injected into LC-MS for analysis. As shown in Figure 3 (trace i), the wild type strain produces **1** as the major metabolite, with **2** and **3** as minor products depending on the culture time. In contrast, the *PdmS* mutant did not produce **1-3**. Instead, a new peak **4** was produced as a dominant product at 44 min (Fig. 3, trace ii). The UV spectrum **4** is similar to that of **1** (Fig. S1), indicating that they have the same chromophore. Its molecular weight was found to be 549, according to the ion peaks [M-H]⁻ at *m/z* 548 and [M+H]⁺ at *m/z* 550.1 in the ESI-MS spectra (Fig. S2). This suggested that **4** is the pradimicin aglycon that has no sugar moieties. We then purified **4**⁷ and recorded its NMR spectra. The ¹H and ¹³C NMR spectra indicated that there are only signals of the pradimicin aglycon in **4**, confirming that it is pradimicinone I. The ¹H and ¹³C NMR signals were assigned based on the 2D NMR spectra including HSQC, HMBC (Fig. S3) and ROESY (Fig. S3), and are shown in Table 1. These data were identical with those of reported for pradimicinone I.⁸ Production of **1-3** by *A. hibisca* revealed that pradimicins with one or two sugar moieties are naturally synthesized, suggesting that the two sugar moieties are successively introduced. Identification of the product of the *PdmS* mutant revealed the function of *PdmS* as the O-GT that is responsible for the introduction of the first sugar moiety, 4',6'-dideoxy-4'-amino-D-galactose or 4',6'-dideoxy-4'-methylamino-D-galactose.

We next sought to identify the O-GT that transfers the D-xylose moiety. *PdmQ* (GenBank accession number ABK58687) is the second putative GT in the *pdm* gene cluster that contains 435 aa. BLAST analysis showed that it is homologous to many GTs that are

proposed to catalyze the transfer of sugar moieties from activated donor molecules to specific aglycons. The closest homologs found in GenBank are two GTs from *Spirillospora albida* (GenBank accession number WP_030163570, 414 aa, 61% identity) and *Thermomonospora curvata* (GenBank accession number WP_012855060, 418 aa, 62% identity), respectively. Again, none of these homologs are identified. Using a similar double crossover approach (Fig. 2A), we constructed a pKC1139-derived disruption plasmid pKN88 (Table S2) after the amplification of a 1224-bp left arm and a 1445-bp right arm. With this plasmid, we obtained a PdmQ mutant of *A. hibisca* P157-2. As shown in Figure 2B (panel ii), the intact *pdmQ* gene is 1.3 kb, while its mutant *pdmQ** is 1.0 kb, confirming that a 0.3-kb fragment has been deleted from *pdmQ*. This mutant was grown in YM medium and the fermentation broth was analyzed by LC-MS. The double crossover mutant of *A. hibisca*/pKN88 did not produce **1** and **3**, but a dominant product with the same retention time as **2** (trace iii of Fig. 3). Further analysis of the UV absorption spectrum (Fig. S1) and MS (Fig. S2) confirmed that this product is indeed pradimicin B (**2**). Thus, disruption of PdmQ led to the production of a major product with only the 4',6'-dideoxy-4'-methylamino-D-galactose moiety, which confirmed the function of this enzyme as the O-GT responsible for transferring the second sugar moiety, D-xylose. **4** was observed as a minor product in this mutant, likely due to the incomplete glycosylation of this aglycon by PdmS.

The structure of **1** contains a 4',6'-dideoxy-4'-methylamino-D-galactose moiety, while that of **3** does not have the N-CH₃ group. A methyltransferase (MT) must be responsible for the methylation of this sugar moiety at the specific 4'-NH₂ group. There are three putative MTs in the *pdm* gene cluster, including *pdmF*, *pdmT* and *pdmO*. BLAST analysis suggested that PdmF and PdmT are two O-MTs, while PdmO is a putative N-MT. PdmO (GenBank accession number ABM21743.1) contains 238 aa. It is homologous to a number of class I MTs that are dependent of S-adenosylmethionine such as the sugar N, N-dimethyltransferases DesVI from *Streptomyces venezuelae* (67% similarity and 53% identity)⁹ and TylM1 from *Streptomyces fradiae* (66% similarity and 52% identity).¹⁰ Both enzymes are involved in the biosynthesis of aminodeoxysugars. Thus, PdmO is a candidate enzyme that catalyzes the specific N-methylation of 4',6'-dideoxy-4'-amino-D-galactose.

To determine the function of PdmO, we constructed an integrative plasmid pKN99 (Table S2) from the pKC1139 vector and a 1.3-kb insert that contains the C-terminal portion of *pdmO* and N-terminal portion of *pdmS* (Fig. 2A). The plasmid pKN99 was introduced into *A. hibisca* through *E. coli* ET12567 (pUZ8002) intergeneric conjugation, as described for the disruption of *pdmS*. The conjugants were selected on ISP4 media with 50 µg mL⁻¹ apramycin at 37°C. Integration of pKN99 into the genome of *A. hibisca* was confirmed by PCR analysis using genome- and vector-specific primers (Table S1). With primer 1 and RV-M, a 1.6-kb fragment was amplified from the genome of *A. hibisca*/pKN99 (Fig. 2B, panel iii, lane 1). Similarly, a 1.6-kb PCR product was obtained with primer 2 and M13-47 from the mutant (lane 2, panel iii, Fig. 2B, panel iii, lane 2). M13-47 and RV-M primers are unique sequences from the vector pKC1139. Consistently, these two 1.6-kb fragments could not be amplified from the wild type (data not shown). Primers 1 and 2 amplified a 1.4-kb band from the genomic DNA of the wild type *A. hibisca*, while no PCR product was obtained from the mutant *A. hibisca*/pKN99 (Fig. 2B, panel iii, lanes 4 and 3). This clearly

shows the insertion of a long segment of DNA between primer 1 and primer 2. Thus, these PCR results confirmed that pKN99 was successfully inserted into the genome of *A. hibisca*.

The fermentation broth of *A. hibisca*/pKN99 in YM medium supplemented with 50 mg L⁻¹ apramycin was analyzed by LCMS. As shown in Figure 3 (trace iv), this strain produced a major product **3** that is slightly polar than **1**. This compound has a UV spectrum (Fig. S1) similar to that of **1**, while ESI-MS revealed that its molecular weight is 826 (Fig. S2), 14 mass units smaller than **1**. It has the same retention time as that of pradimicin C. Thus, it can be concluded that *A. hibisca*/pKN99 produced **3** as the major product, with **1** as the minor product. Given the fact that **3** lacks the N-CH₃, PdmO was deduced to be the dedicated *N*-MT in pradimicin biosynthesis that catalyzes the specific *N*-methylation of the 4,6-dideoxy-4-amino-D-galactose moiety. Integration of pKN99 into the genome of *A. hibisca* did not disrupt *pdmO* (Fig. 2A). Consequently, the synthesis of **1** was still observed. However, insertion of a large fragment upstream of the intact *pdmO* may have influenced the function of the promoter that controls the expression of PdmO. This explains why **3** was generated as the major metabolite and is similar to observations in our previous work on HerF in herboxidiene biosynthesis.¹¹ **4** was also a minor metabolite in this mutant, which might be caused by the incomplete glycosylation of the aglycon.

In summary, using a double crossover recombination approach, we disrupted two putative GT genes in the pradimicin biosynthetic gene cluster, leading to the production of pradimicin analogs with no or one sugar moiety. Based on the corresponding product of the mutants, PdmS and PdmQ were characterized as the GTs that are respectively responsible for the introduction of the first and second sugar moieties to the aglycon **4** (Fig. 4). By inserting a large gene fragment into the genome of *A. hibisca*, we also found that PdmO methylates the amino group of the 4',6'-dideoxy-4'-amino-D-galactose moiety in pradimicin biosynthesis (Fig. 4). Unveiling the roles of these GTs and the *N*-MT further facilitates the understanding and rational engineering of the pradimicin biosynthetic pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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9. *E. coli* ET12567 (pUZ8002) was transformed with pKN82 as the conjugation donor. 100 mL of the culture of *E. coli* ET12567(pUZ8002)/pKN82 or pKN88 was grown in LB broth to an OD₆₀₀ of 0.4–0.6 in the presence of 50 mg L⁻¹ apramycin, 50 mg L⁻¹ kanamycin, and 25 mg L⁻¹ chloramphenicol. The cells were then collected by centrifugation and washed twice with blank LB medium to remove the antibiotics, and then suspended in 0.1 mL of LB medium. *A. hibisca* spores were collected with 2× YT broth after growing for 15 days on YM medium at 30°C. The *A. hibisca* spores were heat shocked at 50°C for 10 min. Serial dilutions of the germinated spore suspension were made and 100 µL of each dilution was mixed with an equal volume of *E. coli* ET12567 (pUZ8002)/pKN82 on MS plates (20 g L⁻¹ D-mannitol, 20 g L⁻¹ soya flower, 20 g L⁻¹ glucose, and 20 g L⁻¹ agar), which were grown for 12 h at 30°C. After incubation, the plates were overlaid with 1 mL of sterile water containing 30 mg L⁻¹ nalidixic acid and 50 mg L⁻¹ apramycin, and incubated at 30°C until the exconjugants appeared. Exconjugants were visible after 4–5 days and started producing spores in 8–10 days.
10. The culture of the double crossover mutant of *A. hibisca*/pKN82 in 500 mL of YM medium was centrifuged and the supernatant was subjected to Diaion HP-20 resin column chromatography, eluted with 0%, 25%, 50%, 75%, and 100% aqueous methanol (v/v) to yield five fractions. HPLC analysis revealed that the 75% fraction contained the target peak. This fraction was further separated by reverse-phase HPLC (Agilent Zorbax SB-C18, 5 µm, 21.2 mm × 150 mm), eluted with 35% aqueous acetonitrile containing 0.1% formic acid, to yield 5.0 mg of **4**.
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Figure 1.
The structures of pradimicins (1-3) and their aglycon (4).

Figure 1.

Disruption of *pdmS*, *pdmQ* and *pdmO* in *A.hibisca*. (A) The single and double crossover strategies used for different genes. (B) Verification of the correct mutants by PCR. (i) Amplification of *pdmS* from the wild type (1.3 kb) and mutant (0.9 kb). M: marker; 1: wild type; 2: *A. hibisca*/pKN82 double crossover mutant. (ii) Amplification of *pdmQ* from the wild type (1.2 kb) and mutant strain (1.0 kb). M: marker; 1: wild type; 2: *A. hibisca*/pKN88 double crossover mutant. (iii) Verification of the insertion of pKN99 into the genome of *A. hibisca*. M: marker; 1: primer 1/RV-M PCR product from the *A. hibisca*/pKN99 single

crossover mutant; 2: primer 2/M13-47 PCR product from the *A. hibisca*/pKN99 single crossover mutant; 3: primer 1/primer 2 PCR product from the *A. hibisca*/pKN99 single crossover mutant; 4: primer 1/primer 2 PCR product from the wild type.

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Figure 3.

HPLC analysis of the products of *A. hibisca* (i), *A. hibisca* PdmS (ii), *A. hibisca* PdmQ (iii), and *A. hibisca*/pKN99 single crossover mutant (iv) at 460 nm. Samples were eluted with 20–35% acetonitrile-water (containing 0.1% trifluoroacetic acid) over 40 min at 1 mL min⁻¹.

Figure 4.
The biosynthetic pathway of **1**.

Table 1¹H (300 MHz) and ¹³C (75 MHz) NMR data for **4** (in DMSO-*d*₆).

No.	δ _H	δ _C
1	-	150.9
2	-	127.1
3	-	137.4
4	7.04 (1H, s)	118.0
4a	-	140.9
5	4.22 (1H, brs)	71.5
6	4.22 (1H, brs)	72.4
6a	-	149.9
7	8.07 (1H, s)	115.6
7a	-	131.2
8	-	187.5
8a	-	110.1
9	-	164.8
10	6.87 (1H, brs)	106.9
11	-	166.0
12	7.21 (1H, brs)	107.6
12a	-	134.3
13	-	185.2
13a	-	115.4
14	-	156.8
14a	-	125.9
14b	-	113.7
15	2.30 (1H, s)	19.2
16	-	167.2
17	4.37 (1H, m)	47.7
18	-	174.2
19	1.30 (1H, d, <i>J</i> = 6.9 Hz)	16.9
11-OMe	3.89 (3H, s)	56.5
9-OH	12.87 (1H, s)	-
16-NH	8.50 (1H, d, <i>J</i> = 6.5 Hz)	-