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Functional analysis of a prenyltransferase gene (*paxD*) in the paxilline biosynthetic gene cluster

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Abstract Paxilline is an indole-diterpene produced by *Penicillium paxilli*. Six genes (*paxB*, *C*, *G*, *M*, *P*, and *Q*) in paxilline biosynthetic gene cluster were previously shown to be responsible for paxilline biosynthesis. In this study, we have characterized *paxD*, which is located next to *paxQ* and has weak similarities to fungal dimethylallyl tryptophan synthase genes. PaxD was overexpressed in *Escherichia coli* and the purified enzyme was used for in vitro analysis. When paxilline and dimethylallyl diphosphate were used as substrates, one major and one minor product, which were identified as di-prenyl paxilline and mono-prenyl paxilline by liquid chromatography–mass spectrometry analysis, were formed. The structure of the major product was determined to be 21,22-diprenylated paxilline, showing that PaxD catalyzed the successive di-prenylation. Traces of both products were detected in culture broth of *P. paxilli* by liquid chromatography–mass spectrometry analysis. The enzyme is likely to be a dimer and required no divalent cations. The optimum pH and temperature were 8.0 and 37 °C, respectively. The *Km* values were calculated as 106.4±5.4 μM for paxilline and 0.57±0.02 μM for DMAPP with a *kcat* of 0.97±0.01/s.

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Introduction

Isoprenoid compounds found in nature—with over 50,000 known examples—contain industrially useful compounds such as flavors, antibiotics, and plant hormones, among others (Christianson 2007; Connolly and Hill 1992; Dewick 2002). In some cases, isoprenoids are attached to other moieties, such as polyketide (Edwards and Gerwick 2004; Lo et al. 2012), indole/tryptophan (Steffan et al. 2009), (iso)flavonoid (Tahara and Ibrahim 1995), and phenazine moieties (Izumikawa et al. 2010; Saleh et al. 2009); Prenylated indole compounds, most of which are produced by filamentous fungi, are examples of this type (Li 2010). These compounds are derived from polyprenyl diphosphates and tryptophan or its derivatives and show diverse chemical structures and biological activities (Dalziel et al. 2005; Gonzalez et al. 2003; Singh et al. 2004). Among prenylated indole compounds, indole-diterpenes are unique from the following viewpoints: geranylgeranyl diphosphate (GGDP) is used as a prenyl donor in contrast to other prenylated indoles, for which dimethylallyl diphosphate (DMAPP) is the usual donor, and indole/indole-3-glycerol phosphate is used as the prenyl acceptor instead of tryptophan and its derivatives (Byrne et al. 2002; Saikia et al. 2008; Uchida et al. 2006; Tagami et al. 2013).

Pioneering studies of biosynthetic genes and enzymes for indole-diterpenes were carried out with *P. paxilli*, a paxilline producer. They first identified 17 clustered genes in a genomic locus (Young et al. 2001). They later showed that *paxG*, *paxM*, *paxB*, and *paxC* were required for paspaline biosynthesis by expression of these genes in a mutant deleted for the *pax* gene cluster (Saikia et al. 2006). Paspaline was

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shown to be successively oxidized by a cytochrome P450 gene, *paxP*, to give 13-desoxypaxilline, followed by an additional oxidation by *paxQ*, to give paxilline (Fig. 1) (Saikia et al. 2007). Very recently, we have characterized the detailed function of each of these *pax* genes (*paxG*, *paxC*, *paxM*, *paxB*, *paxP*, and *paxQ*) by heterologous expression, in vitro assay with recombinant enzymes and biotransformation (Fig. 1) (Tagami et al. 2013). We confirmed that a transformant of *Aspergillus oryzae* carrying *paxG* and *paxC* produced geranylgeranyl indole (GGI). Introduction of additional *paxM*, *paxB*, and *paxP*/*paxQ* genes into the transformant resulted in the production of monoepoxide GGI, paspaline, and paxilline, respectively. By in vitro experiments using recombinant enzyme, PaxC was confirmed to be a prenyltransferase that forms GGI by transfer of GGDP to both indole and indole-3-glycerol phosphate. Moreover, epoxidation of GGI, cyclization of monoepoxide GGI, and two successive hydroxylations of paxilline were verified by biotransformation experiments using *A. oryzae* carrying *paxM*, *paxB*, and *paxP*/*Q* genes, respectively (Tagami et al. 2013).

However, the functions of the other *pax* genes previously identified in the paxilline biosynthetic gene cluster remained unclear. In this study, we focused on the putative prenyltransferase gene *paxD*, which is located next to the *paxQ* gene, and examined whether it participates in the biosynthesis of paxilline derivatives.

Materials and methods

General

Sequence analysis of polymerase chain reaction (PCR) fragments was performed by the dideoxy chain termination method with an automatic DNA sequencer (LI-COR, model 4000L, Lincoln, NE, USA). Cell disruption was performed with an Ultrasonic Disruptor (TOMY, UD-200, Tokyo, Japan). Analysis of the samples during protein purification was performed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were visualized by Coomassie brilliant blue staining. Protein concentration was determined by the Bradford method (Bradford 1976) with bovine serum albumin as a standard.

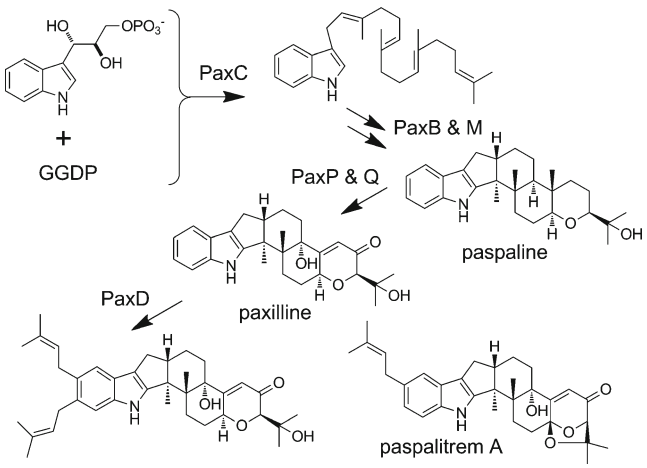

Fig. 1 Biosynthetic scheme for paxilline. Structures of 21,22-diprenylated paxilline and paspalitrem A are also shown

Strain and plasmids

P. paxilli ATCC26601 was used as the experimental material (Young et al. 1998). Cultivation and cDNA preparation were performed as described previously (Tagami et al. 2013). Plasmids from *Escherichia coli* were prepared using a Qiagen plasmid kit (Hilden, Germany). All restriction enzymes, T4 ligase, and calf intestinal alkaline phosphatase were obtained from Toyobo (Osaka, Japan), and used according to the manufacturer's instructions. Transformation of *E. coli* with plasmid DNA by electroporation was performed under standard conditions with a BTX ECM 600 electroporation system (Biotechnologies and Experimental Research, San Diego, CA, USA).

Cloning, overexpression, and purification of PaxD

The cDNA carrying the *paxD* gene (GenBank; AAK11526) was amplified by PCR using gene-specific primers: 5′-TTGGTACCATGCAAAGCGATGAGTTGCAAATCCCACC-3′ and 5′-TTCTGCAGCTATGCCTTGAAAAGCTTAGGAGCCCATAC-3′. After subcloning and sequence confirmation, a 1.3-kb fragment obtained by *Kpn*I and *Pst*I digestion was ligated into pQE30 (Qiagen). *E. coli* M15 carrying pQE30 (a control) and pQE30-PaxD were separately grown in L-broth supplemented with 100 μg/mL ampicillin. The strain was grown at 37 °C until optical density (600 nm) reached 0.5 and then isopropyl β-D-thiogalactopyranoside (0.5 mM) was added to the culture, followed by additional cultivation at 18 °C for 18 h. The harvested cells were suspended in buffer containing 50 mM tris(hydroxymethyl)aminomethane (Tris)–HCl (pH 8.0), 0.5 M NaCl, and 10 mM imidazole, and then disrupted by sonication. The debris was removed by centrifugation, and the supernatant was applied on a Ni^{2+}-nitrilotriacetic acid agarose column (Qiagen) equilibrated with buffer containing 50 mM Tris–HCl (pH 8.0), 0.5 M NaCl, and 10 mM imidazole. The column was washed with equilibration buffer and then eluted with buffer containing 50 mM Tris–HCl (pH 8.0), 0.5 M NaCl, and 0.5 M imidazole. The eluent was desalted using an Amicon apparatus (Millipore, Bedford, MA, USA). After the purity of the recombinant enzyme was checked by SDS-PAGE, it was used for in vitro assay. For molecular mass and subunit structure determination of PaxD, the purified

enzyme was loaded onto a HiLoad 26/60 Superdex 75 pg gel-filtration column (Amersham Biosciences, Piscataway, NJ, USA) and eluted with a buffer containing 50 mM Tris–HCl (pH 8.0) and 10 mM NaCl. The retention volume of PaxD was compared with those of the marker proteins. The markers applied to the column were aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa) (GE Healthcare, Little Chalfont, UK).

In vitro assay of prenyltransferases

The standard assay mixture for PaxD contained in a final volume of 100 μL, 0.25 mM of prenyl acceptors, 0.25 mM of DMAPP, 50 mM Tris–HCl (pH 8.0), and a suitable amount of PaxD. This mixture was incubated at 30 °C overnight and the reaction was stopped by the addition of 100 μL methanol. The products were analyzed by high performance liquid chromatography (HPLC). Analytical conditions were as follows: Merck Mightysil RP-18GP Aqua column (250×4.6 mm) (Kanto Chemicals, Tokyo, Japan); mobile phase of acetonitrile in water (0 to 25 min, 70 % acetonitrile; 25 to 40 min, 70 to 100 %; 40 to 50 min, 100 %); flow rate, 1.0 mL/min; detection, 205 nm.

The following compounds were used for examination of substrate specificity: benzyl D -glucopyranoside, phenyl α-D-glucopyranoside, β-naphthyl α-D-glucopyranoside, naringenin-7-glucoside, polydatin, cyclodipeptides (cyclo-L-Trp-L-Tyr, cyclo-L-Pro-L-Tyr, cyclo-L-His-L-Phe, cyclo-L-Phe-L-Pro, cyclo-L-Phe-L-Trp, cyclo-L-Phe-L-Leu, all of which were kindly provided by Dr. H. Kanzaki of Okayama University, Japan), hydroxynaphthalenes (1-naphthol, 1,3-dihydroxynaphthalene, 2,6-dihydroxynaphthalene, 2,7-dihydroxynaphthalene, 3,7-dihydroxy-2-naphtholic acid), indole, L-tryptophan and L-tyrosine.

The steady-state kinetic parameters were determined by fitting to the Michaelis–Menten equation. The assay was linear with respect to protein concentration up to 1 μg for 20 min incubation and no substrate inhibition was observed with paxilline or DMAPP up to 1.0 mM of each substrate. The assays for determination of the kinetic parameters of paxilline contained in a final volume of 100 μL, 50 mM Tris–HCl (pH 8.0), 0.1 mM DMAPP, 0.2 μg of PaxD, and 20 μM to 0.5 mM paxilline. This mixture was incubated at 30 °C for 10 min. When the concentration of paxilline was fixed at 1.0 mM, the concentration of DMAPP was varied from 0.1 to 20 μM.

Metal dependency of PaxD

Divalent metal ions (5 mM of Mg^{2+}, Ca^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+}, and Co^{2+}), or 5 mM ethylenediamine-*N*,*N*,*N*',*N*'-tetra acetic acid (EDTA) were added to the standard reaction mixture.

Liquid chromatography–electrospray ionization mass spectrometry analysis

Products formed by in vitro assays and accumulated in culture broth of *P. paxilli* were analyzed by liquid chromatography–electrospray ionization mass spectrometry (LC/ESI-MS) (Waters ACQUITY UPLC equipped with SQD2). The analytical conditions were as follows: Waters ACQUITY UPLC column (2.1×50 mm); column temperature, 40 °C; detection, positive mode; mobile phase, 0.1 % formic acid:acetonitrile=30:70 at 5 min, and a linear gradient to 50:50 for an additional 30 min; flow rate, 0.3 ml/min; cone voltage, 30 V for total and selected ion chromatograms and 50 V for fragmentation analysis.

Structural analysis of the reaction product formed from paxilline and DMAPP

The reaction products using paxilline and DMAPP were fractionated with a preparative HPLC. Then, ^{1}H- and ^{13}C-NMR spectra were recorded on Bruker AMX-500 spectrometer (Table S1). NMR δ_H ($CDCl_3$, 500 MHz) (Fig. S3) 1.02 (s, 3H), 1.28 (s, 3H), 1.29 (s, 3H), 1.30 (s, 3H), 1.43 (dd, *J*=4.6, 13.2 Hz, 1H), 1.65 (d, *J*=12.7 Hz, 1H), 1.71 (s, 3H), 1.74 (s, 6H), 1.75 (s, 3H), 1.78 (m, 1H), 1.88 (m, 1H), 2.02 (m, 1H), 2.06 (m, 1H), 2.32 (m, 1H), 2.41 (dd, *J*=11.0, 13.0 Hz, 1H), 2.71 (dd, *J*=6.2, 13.0 Hz, 1H), 2.79 (m, 1H), 2.82 (m, 1H), 3.39 (d, *J*=6.9 Hz, 4H), 3.72 (d, *J*=1.8 Hz, 1H), 4.85 (t, *J*=9.2 Hz, 1H), 5.29 (t, *J*=6.9 Hz, 1H), 5.30 (t, *J*=6.9 Hz, 1H), 5.88 (d, *J*=1.7 Hz, 1H), 7.10 (s, 1H), 7.21 (s, 1H), 7.56 (s, 1H). NMR δ_C ($CDCl_3$, 125 MHz) (Fig. S4) 16.1, 17.8, 17.9, 19.7, 20.9, 24.2, 25.8 (×2), 26.6, 27.3, 28.0, 28.4, 31.8, 32.0, 34.4, 43.2, 49.4, 50.7, 72.4, 72.6, 77.4, 83.3, 111.4, 117.1. 118.4, 119.6, 123.4, 123.8, 124.2, 130.0, 131.4, 131.6, 132.8, 138.8, 151.1, 168.2, 199.3. High-resolution electrospray ionization mass spectrometry (HR-ESI-MS): calcd. for $C_{37}H_{50}NO_4$ $[M+H]^+$: 572.3734, found: 572.3754.

Results

Functional analysis of PaxD

The detailed functions of each of the *paxG*, *paxC*, *paxM*, *paxB*, *paxP*, and *paxQ* genes in the biosynthesis of paxilline were analyzed previously (Saikia et al. 2006; Saikia et al. 2007; Tagami et al. 2013). However, additional genes such as *paxD* and *paxO*, the gene products of which are similar to dimethylallyl tryptophan synthases and oxidoreductases, respectively, exist in the flanking region of the paxilline biosynthetic gene cluster (Young et al. 2001). In particular, the *paxD* gene is located just next to the *paxQ* gene, which catalyzes the hydroxylation of 13-desoxypaxilline to give

paxilline. Therefore, we assumed that the *paxD* gene might be involved in a tailoring reaction after the formation of paxilline. To examine this possibility, recombinant PaxD enzyme was prepared and used for in vitro assay.

A cDNA of *paxD* was amplified by the rapid amplification of cDNA ends method based on the nucleotide sequence of the cDNA reported by Scott et al. (Young et al. 2001) (GenBank; AAK11526). The predicted gene product consisted of 438 amino acids, which was the same as previously reported. In a BLAST search, even more than 10 years later, the most homologous enzyme to PaxD was the *atmD* product (32 % amino acid identity, Fig. S1) as reported by Scott et al. *atmD* is located in the aflatrem biosynthetic gene cluster in *Aspergillus flavus* although its function remains unclear (Nicholson et al. 2009). The PaxD cDNA was cloned into the pQE30 vector for protein expression in *E. coli*. His-tagged PaxD recombinant enzyme was successfully expressed in a soluble form under the conditions described in Materials and Methods. Purified enzymes were obtained by Ni^{2+} column chromatography and successive desalting with Amicon Ultra devices. The obtained recombinant PaxD, which had a calculated molecular mass of 47 kDa, was subjected to SDS-PAGE analysis to confirm its molecular size and purity (Fig. 2a). The recombinant enzyme was then subjected to gel filtration to estimate its subunit structure. As shown in Fig. 2b, a peak with a calculated molecular mass of 105 kDa was detected, suggesting that PaxD forms a homodimer structure.

Catalytic activity was then examined using purified recombinant enzymes. Considering that the final product formed by PaxG, PaxC, PaxM, PaxB, PaxP, and PaxQ is paxilline and that PaxD has weak similarities to dimethylallyl tryptophan synthases that use DMAPP as a substrate (Balibar et al. 2007; Ding et al. 2008; Grundmann et al. 2008; Grundmann and Li 2005; Kremer and Li 2010; Kremer et al. 2007; Liu and Walsh 2009; Noike et al. 2012; Unsold and Li 2005; Unsold and Li 2006; Yin et al. 2009; Yin et al. 2007; Yin et al. 2010; Zou et al. 2010), we thought that PaxD might catalyze a prenylation of paxilline. The purified recombinant PaxD was therefore incubated with paxilline and DMAPP. A major product (Fig. 3, peak B) and a minor product (Fig. 3, peak A) were specifically detected by HPLC analysis. Total ion chromatograms obtained by LC/ESI-MS analysis showed two specific peaks with molecular masses corresponding to mono-prenylated and diprenylated paxilline (Fig. S2). Moreover, selected ion chromatograms and their mass spectra strongly suggested that one product was mono-prenylated paxilline and the other diprenylated paxilline (Fig. 4a-d). Since the yield of the minor product was low, we determined the exact structure of the major product. HR-ESI-MS of the major product (peak B) indicated the molecular formula $C_{37}H_{50}NO_4$, supporting production of diprenylated paxilline. ^{1}H-NMR spectra showed new signals for regular prenyl moieties at δ=5.30 (t, *J*=6.9 Hz, 1H), δ=5.29 (t, *J*=6.9 Hz, 1H), and δ=3.39 (d, *J*=6.9 Hz, 4H) concomitant with two singlet signals (δ=7.21 and δ=7.10) without *o*- and *m*-coupling, indicating the substitution of the prenyl moieties at C-21 and C-22 positions on the indole moiety. Finally, extensive NMR data analysis, including correlation spectroscopy (Fig. S5), heteronuclear single quantum coherence (Fig. S6), heteronuclear multiple quantum coherence (Fig. S7), and nuclear overhauser effect spectroscopy (Fig. S8) proved the structure as 21,22-diprenylated paxilline. Since fungal dimethylallyl tryptophan synthases usually catalyze the formation of mono-prenylated compounds as the main product, and diprenylated compounds were reported to be produced as minor products by a few enzymes such as AnaPT and 7-DMATS (Yu et al. 2011), the reaction catalyzed by PaxD is unique.

Prenylated paxillines are natural products produced by *P. paxilli*

Since PaxD catalyzed the prenylation of paxilline in vitro, we then examined whether PaxD functioned in vivo by searching for mono-prenylated paxilline and 21,22-

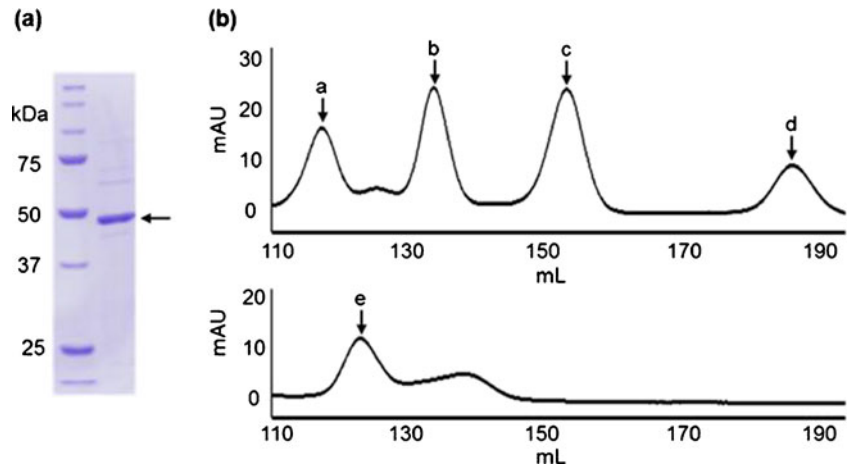

Fig. 2 Purified PaxD was analyzed by SDS-PAGE (**a**) and gel-filtration chromatography (**b**). **a** molecular mass markers (*lane 1*) and purified PaxD (*lane 2*). **b** elution profiles of the standard proteins [aldolase (*a* 158 kDa), albumin (*b* 67 kDa), ovalbumin (*c* 43 kDa) and chymotrypsinogen A (*d* 25 kDa); top] and purified PaxD (bottom, *e*)

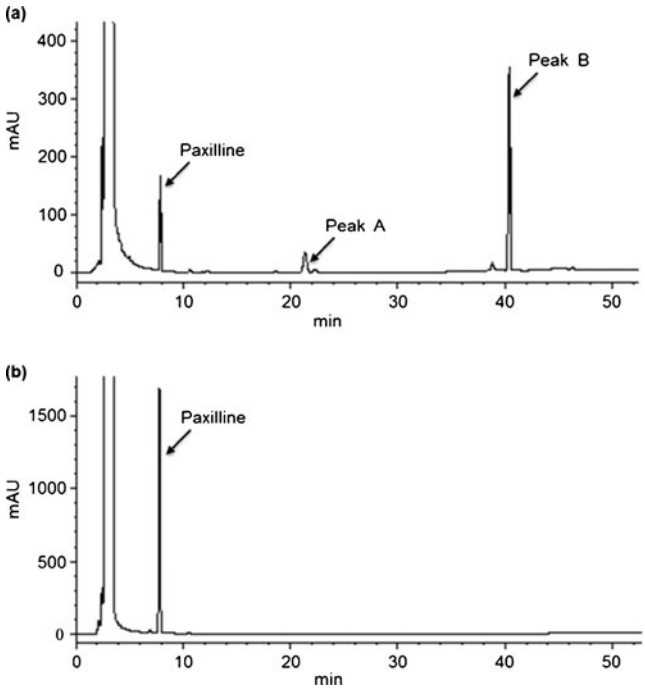

Fig. 3 Analysis of the reaction products formed from paxilline and DMAPP. The reaction products formed with (**a**) and without (**b**) PaxD were analyzed by HPLC

diprenylated paxilline in culture broth of the paxilline producer. After *P. paxilli* was cultivated under standard conditions, the supernatant of the culture broth obtained by centrifugation was extracted with ethyl acetate and evaporated in vacuo (Weedon and Mantle 1987). The sample thus obtained was analyzed by LC/ESI-MS. For mono-prenylated paxilline, a peak with a molecular mass of 504.27, which corresponded to $[M + H]^+$ of mono-prenylated paxilline, was detected at the same retention time as in the in vitro assay (Fig. 4e) by selected ion chromatogram. The same fragmentations as those of the in vitro product were also observed (Fig. S2e). Moreover, a specific fragment of 198.09, which had been observed as a fragment of paspalitrem A by LC/ESI-MS analysis, was detected. Since the structures of the A to E rings of paspalitrem A and 21-prenylated paxilline are the same (Fig. 1) (Uhlig et al. 2009), this spectrum (198.09) was suggested to result from the same fragmentation as that of paspalitrem A. For diprenylated paxilline, a peak with the molecular mass of 21,22-(di)prenylated paxilline (572.32) was detected in the same manner as that of the in vitro sample (Fig. 4f, h). A fragment of 266.20, which probably resulted from the same fragmentation pattern as that of mono-prenylated paxilline, was also observed (Fig. S2f). These results suggested that both the mono- and diprenylated paxillines existed in culture broth of *P. paxilli* and that the final fermentation product governed by the *pax* gene cluster in *P. paxilli* might be the prenylated paxillines rather than paxilline, though their productivities were estimated to be approximately 0.5 % of paxilline by LC/ESI-MS.

Biochemical characterization of PaxD

The substrate specificity of the PaxD enzyme was investigated. For the prenyl acceptor, compounds related to indole-diterpene biosynthesis, such as tryptophan, indole or indole-3-glycerol phosphate, and GGI were used. However, no products were formed with DMAPP as the prenyl donor. We also used several cyclodipeptides and hydroxynaphthalenes as substrates since they were reported to be utilized by many fungal prenyltransferases (Grundmann and Li 2005; Yin et al. 2007; Yin et al. 2010; Yu et al. 2011; Zou et al. 2010). However, no products were formed. We next examined the substrate specificity of the prenyl donors. Aside from DMAPP, geranyl diphosphate, farnesyl diphosphate, and GGDP were examined. However, no products were formed with paxilline as the prenyl acceptor.

The biochemical properties of the enzyme were investigated using paxilline and DMAPP as the substrates. Under the conditions described in the Materials and Method Section, product formation was optimal at 37 °C and pH 8.0 (Figs. S9 and S10, respectively). Unusually, the enzyme showed activity over broad pH range (pH 5 to 10). Although PaxD lacked a (N/D)DXXD motif, which is required for binding of divalent cations (Koyama et al. 1996; Tarshis et al. 1994), some prokaryotic prenyltransferases without the motif require divalent cations for activity (Ozaki et al. 2009; Pojer et al. 2003). Therefore, the effects and dependence of divalent metal ions on the PaxD activity were tested at a concentration of 5 mM (Fig. S11). The enzyme showed similar activity regardless of the presence of 5 mM EDTA, suggesting that it did not require Mg^{2+} for its activity. In contrast, the PaxD activity was decreased to less than 50 % of the control experiment (without divalent cations) by the addition of Co^{2+}, Cu^{2+}, and Zn^{2+}.

The kinetic parameters of PaxD were also investigated. The enzyme reaction followed Michaelis–Menten kinetics. By using Hanes–Woolf plots, the *Km* values were calculated as 106.4±5.4 μM for paxilline, a plausible substrate for PaxD, and 0.57±0.02 μM for DMAPP. The *k*cat values were calculated as 0.97±0.01/s (Fig. S12).

Discussion

In this study, we demonstrated that the *paxD* gene in *P. paxilli* encodes a prenyltransferase that catalyzes successive attachment of DMAPP to positions 21 and 22 of paxilline. Since cDNA of the *paxD* gene was obtained, as previously reported, and the prenylated paxillines existed in culture broth of *P. paxilli*, the *paxD* gene was functionally expressed in vivo, showing that *paxD* is a member of the

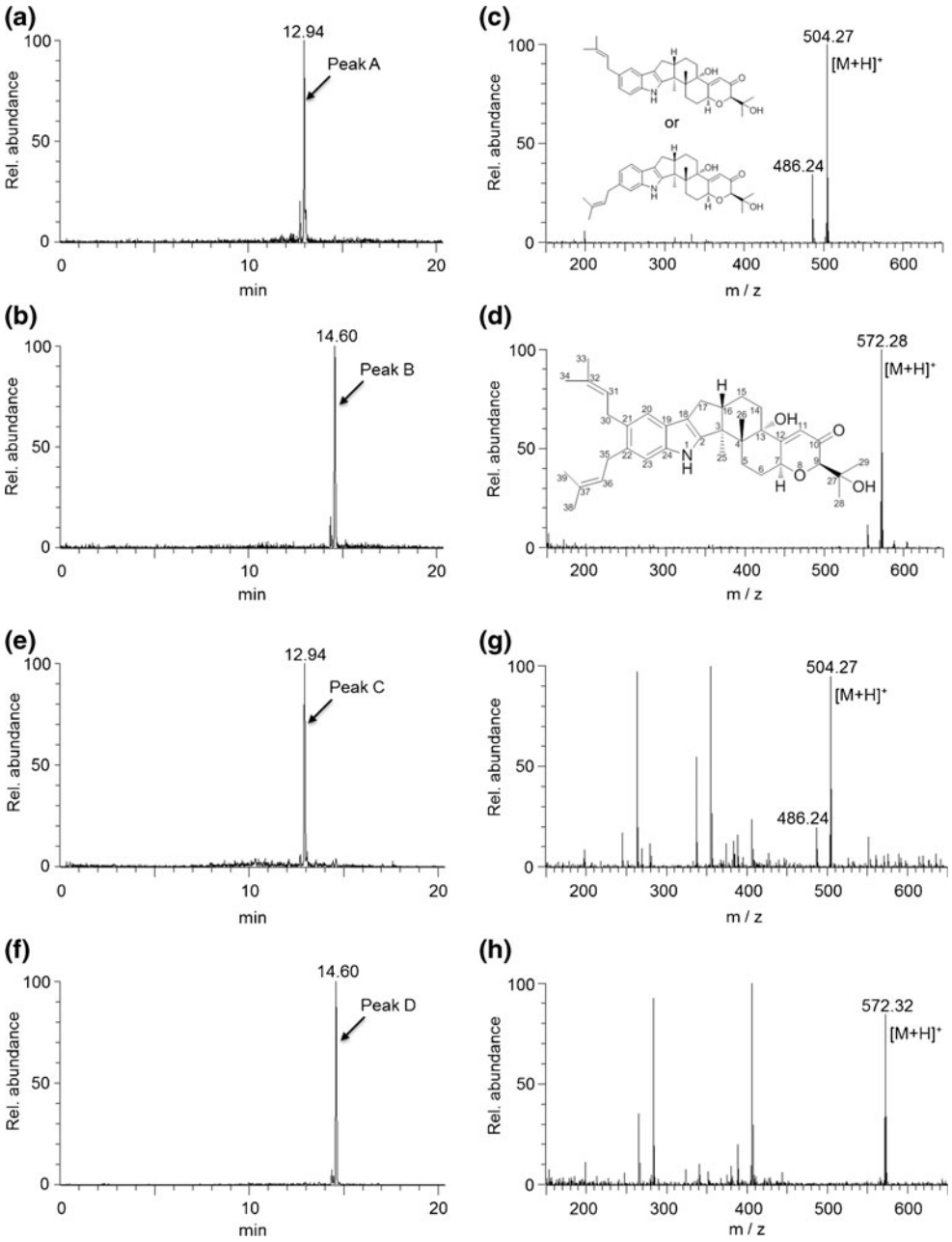

Fig. 4 LC/ESI-MS analysis of the prenylated paxilline formed by in vitro assay (**a**-**d**) and accumulated in culture broth of *P. paxilli* (**e**-**h**). Selected ion chromatograms (**a**, **b**) and spectra (**c**, **d**) for mono-prenylated paxilline (**a**, **c**) and diprenylated paxilline (**b**, **d**). Selected ion chromatograms (**e**, **f**) and mass spectra (**g**, **h**) for mono-prenylated paxilline (**e**, **g**) and diprenylated paxilline (**f**, **h**) in samples from culture broth of *P. paxilli*. Masses of 504 and 572, which correspond to $[M + H]^+$ of mono-prenylated and diprenylated paxilline, were selected. Structures of 21,22-diprenylated paxilline and 21- or 22-prenylated paxilline are also shown

pax gene cluster. However, the estimated amounts of the prenylated paxillines were quite low when compared to paxilline. This would explain why there were no reports of the prenylated paxillines after the discovery of paxilline. The calculated *k*cat/*Km* value of PaxD for paxilline was 9.2 S^{-1} mM^{-1}. This value is slightly lower than that of PaxC (28.2 S^{-1} mM^{-1} for indole-3-glycerol phosphate), which catalyzes the transfer of GGDP to indole-3-glycerol phosphate to give GGI, the first intermediate in paxilline biosynthesis (Tagami et al. 2013). The low catalytic activity of PaxD might be one of the reasons for the accumulation of paxilline in culture broth of *P. paxilli*.

In the in vitro assay, two products (Fig. 3, peaks A and B) were detected with paxilline and DMAPP as substrates. We could not determine the structure of the minor compound because of the low yield. However, considering that the products formed by PaxD were 21,22-diprenylated paxilline and mono-prenylated paxilline and that the fragment spectrum (198.09), which had been shown to result from fragmentation in the C ring of paspalitrem A (Uhlig et al. 2009), of the mono-prenylated paxilline was also detected by LC/ESI-MS analysis (Fig. S2e), the mono-prenylated compound would be either 21-prenylated paxilline or 22-prenylated paxilline. In that case, PaxD catalyzes a stepwise

di-prenylation via a mono-prenylated intermediate. Since fungal dimethylallyl tryptophan synthases usually catalyze the transfer of one molecule of DMAPP, the di-prenylation catalyzed by PaxD is unique. We were not able to estimate the differences in the reaction mechanism between mono-prenylation and di-prenylation. However, comparisons of a crystal structure of PaxD with those of fungal enzymes catalyzing mono-prenylation, such as FtmPT1 (Jost et al. 2010), might give us a clue.

PaxD did not react with cyclodipeptides and hydroxynaphthalenes, which were accepted by other fungal indole prenyltransferases. Our results are in good agreement with the low amino acid sequence similarities between PaxD and the fungal prenyltransferases (17–22 % identities, Fig. S13). Moreover, the very low *Km* value for DMAPP reasonably supports the result that farnesyl diphosphate and GGDP were not accepted as substrates. On the other hand, the *Km* value for paxilline was calculated to be higher than for DMAPP, suggesting that paxilline-related compounds, such as paspaline, 13-desoxypaxilline, lolitrems (Saikia et al. 2012), and terpendoles (Motoyama et al. 2012), might be accepted by PaxD. However, we did not have access to such compounds and could not examine this possibility.

In conclusion, we showed that *paxD* gene, which is located in paxilline biosynthetic gene cluster in *P. paxilli*, encoded the prenyltransferase catalyzing successive transfer of two molecules of DMAPP into paxilline to yield 21,22-diprenylated paxilline. The di-prenylation reaction is rarely catalyzed by indole prenyltransferase family, and then, the enzymatic properties were investigated. We could detect trace amounts of the product in culture broth of *P. paxilli* by LC/ESI-MS analysis, showing that the final fermentation product governed by the *pax* gene cluster in *P. paxilli* is the prenylated paxillines rather than paxilline, though their productivities were low.

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