

Characterization of the polyene macrolide P450 epoxidase from *Streptomyces natalensis* that converts de-epoxypimaricin into pimaricin

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The biosynthesis of the antifungal agent pimaricin by *Streptomyces natalensis* has been proposed to involve a cytochrome P450 encoded by the gene *pimD*. Pimaricin is derived from its immediate precursor de-epoxypimaricin by epoxidation of the C-4–C-5 double bond on the macrolactone ring. We have overproduced PimD with a N-terminal His₆ affinity tag in *Escherichia coli* and purified the enzyme for kinetic analysis. The protein showed a reduced CO-difference spectrum with a Soret maximum at 450 nm, indicating that it is a cytochrome P450. Purified PimD was shown to catalyse the *in vitro* C-4–C-5 epoxidation of 4,5-de-epoxypimaricin to pimaricin. The enzyme was dependent on NADPH

for activity with optimal pH at 7.5, and the temperature optimum was 30 °C. The k_{cat} value for the epoxidation of de-epoxypimaricin was similar to the values reported for other macrolide oxidases. Enzyme activity was inhibited at high substrate concentration. This is the first time that a polyene macrolide P450 mono-oxygenase has been expressed heterologously and studied. The unique specificity of this epoxidase should be useful for the oxidative modification of novel polyene macrolide antibiotics.

Key words: cytochrome P450 mono-oxygenase, epoxidase, pimaricin, polyene, polyketide synthase.

INTRODUCTION

The biosynthesis of macrolides often requires the tailoring action of specific oxidases in order to construct the final bioactive compound. Such oxidases belong to a family of enzymes that is one of the most widely distributed in nature, the cytochromes P450. These are *b*-type cytochromes that carry out oxygenation reactions on an enormous array of substrates [1], among them the polyketide precursors of macrolide antibiotics. Examples include reactions involved in the biosynthesis of erythromycin [2,3], tylosin [4], oleandomycin [5], narbomycin [6,7], epothilone [8,9], amphoterin [10], nystatin [11], pimaricin [12,13] and rapamycin [14], among others. These reactions typically occur during the late stages of the biosynthesis, once the macrolide ring has been constructed by the polyketide synthase, and constitute an important contribution for biological activity [6,15]. However, although cytochromes P450 using polyketide structures as substrates are common, there are considerable variations in the substrates that are utilized, and the positions of the resulting hydroxy and/or epoxide substituents to such compounds introduced by these enzymes. This diversity may be exploited to obtain novel products by bioconversions. It is, therefore, of great importance to characterize additional macrolide mono-oxygenases in order to target the increasing number of novel polyketides obtained by combinatorial techniques.

Thus far, the erythromycin hydroxylases EryF and EryK from *Saccharopolyspora erythraea* [16,17], the methymycin/picromycin hydroxylase PicK (PikC) from *Streptomyces venezuelae* [6,7] and the epothilone epoxidase EpoK [8], are the only enzymes from this family to be expressed, purified and characterized. The first three enzymes catalyse hydroxylation of 12- or/and 14-membered macrolactones. While EryF and EryK exhibit strict substrate specificity and are responsible for the hydroxylation at single positions of the 14-membered erythromycin aglycone [16,17],

PicK has broader substrate specificity, accepting both 12- and 14-membered ring macrolides as substrates, as well as catalysing the addition of a hydroxy group at two different positions in the case of the 12-membered macrolactone [7,18]. EpoK specifically converts deoxyepothilone into epothilone, a 16-membered macrolactone [8].

Pimaricin is a 26-membered polyene macrolide antifungal antibiotic [19], which is widely used for the treatment of fungal keratitis, and also in the food industry in order to prevent mould contamination of cheese and other non-sterile foods (i.e. cured meat, sausages, ham etc.). The biosynthetic gene cluster for pimaricin has been characterized [12,13,15,20]. Insertional inactivation of one of the genes of the pimaricin cluster, *pimD*, generated a mutant that accumulated a glycosylated pimaricin precursor, 4,5-de-epoxypimaricin [15], thus suggesting that this gene codes for a mono-oxygenase that is responsible for the introduction of the epoxy group at C-4–C-5 of the pimaricin molecule. In the present paper, we report the heterologous expression of PimD in *Escherichia coli* and the *in vitro* characterization of this enzyme as the epoxidase that transforms de-epoxypimaricin into pimaricin. The availability of the purified PimD enabled measurement of kinetic parameters of the reaction catalysed by the enzyme and comparison with the oxidations performed by the 12- and 14-membered macrolactone hydroxylases. The present study constitutes the first for a polyene macrolide, and for any other large-size ring macrolactone.

EXPERIMENTAL

Bacterial strains and cultivation

Streptomyces natalensis A.T.C.C. 27448 was routinely grown in YEME (yeast extract–malt extract) medium [21] without sucrose. Sporulation was achieved in TBO (tomato paste–oat flakes)

Abbreviations used: BCIP, 5-bromo-4-chloroindol-3-yl phosphate; IPTG, isopropyl β -D-thiogalactoside; LC/MS, liquid chromatography/mass spectrometry; Ni-NTA, Ni²⁺-nitrilotriacetate; TBS, Tris-buffered saline.

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medium [12] at 30 °C. For pimaricin production, the strain was grown in YEME without sucrose. The same media were supplemented with thioestrepton when used for *St. natalensis* 6D4 growth and/or de-epoxypimaricin production [15]. *E. coli* strain DH5 α was used as a host for DNA manipulation. *E. coli* BL21 (DE3) was used for expression studies.

Plasmids and DNA manipulation procedures

PBluescript was used as the routine cloning vector, and pQE-30 (Qiagen) was the vector used to construct the PimD expression plasmid, pMVM5. Plasmid, cosmid and genomic DNA preparation, DNA digestion, fragment isolation, Southern hybridization, and transformation of *E. coli* were performed by standard procedures [22]. PCRs were carried out using *Pfu* DNA polymerase as described by the enzyme supplier (Stratagene). DNA sequencing was accomplished by the dideoxynucleotide chain-termination method using the PerkinElmer Amplitaq Gold Big Dye-terminator sequencing system with an Applied Biosystems model 310 sequencer (Foster City, CA, U.S.A.).

Construction of PimD *E. coli* expression plasmid

The *pimD* gene was amplified for insertion into the His₆-tag expression vector pQE-30 using PCR. The forward primer used (5'-CCCATGACCGCCGATCCCACGACCTGCC-3') introduced a unique *Bam*HI site at the 5' end of the gene, while the reverse primer (5'-GTCCTCCCCGCGCTGCAGCCCCAG-3') carries a *Pst*I site 36 nucleotides downstream from the TGA translational stop codon. The amplified DNA fragment was digested with *Bam*HI and *Pst*I, and was cloned into the same sites of pQE-30 to generate pMVM5. The amplified DNA fragment was sequenced from the expression vector in order to discard any mistakes introduced by the DNA polymerase.

Expression and purification of His₆-PimD

E. coli cells were grown at 30 °C in 100 ml of LB (Luria-Bertani) medium containing 1% (w/v) glycerol and 100 μ g/ml ampicillin until a D_{600} of between 0.7 and 0.9 was reached, and then induced by adding IPTG (isopropyl β -D-thiogalactoside) to a final concentration of 0.5 mM, and grown for an additional 3 h. Cells were harvested, washed with 0.9% (w/v) NaCl, resuspended in binding buffer (20 mM Tris/HCl, pH 8.0, 500 mM NaCl and 20 mM imidazole) and lysed by sonication using an ultrasonic processor XL apparatus (Misonix, Farmingdale, NY, U.S.A.). The insoluble material was separated by centrifugation at 40 000 *g* for 30 min, and the soluble fraction was applied to a FPLC Ni-NTA (Ni²⁺-nitrilotriacetate)-agarose (Qiagen) HR5/5 column equilibrated with binding buffer. The column was washed with 25 ml of binding buffer, and the protein was eluted with a 15 ml linear gradient (20–500 mM imidazole in binding buffer). Protein elution was monitored at 280 nm, and the presence of PimD was assessed by SDS/PAGE (12% gels). Fractions containing the PimD protein were pooled and dialysed against storage buffer (45 mM Tris/HCl, pH 7.5, 0.1 mM EDTA, 0.2 mM dithiothreitol and 10% glycerol). Enzyme concentration and yield were determined by the Bradford method [23] using BSA as a standard.

Immunoblot analysis

Cell-free extracts were run on SDS/12% polyacrylamide gels and transferred on to nylon membranes using standard techniques. Membranes were then blocked by incubation at room temperature (22 °C) in 5% (w/v) skimmed dried milk in TBS (Tris-buffered

saline solution) for 1 h. After washing, blots were successively incubated with mouse monoclonal anti-His-tag antibodies (Qiagen), goat anti-mouse immunoglobulin-alkaline phosphatase conjugate (Sigma) and BCIP (5-bromo-4-chloroindol-3-yl phosphate)-Nitro Blue Tetrazolium substrate in alkaline phosphatase buffer (100 mM NaCl, 5 mM MgCl₂ and 20 mM Tris/HCl, pH 9.5). A triple wash with TBS containing 2 g/l Tween 20 was performed between incubations, except for the last one in which we used alkaline phosphatase buffer just before the addition of the substrate solution. Colour development was stopped by washing with tap water.

Spectral analysis

Spectra of the purified PimD in storage buffer were recorded from 300 to 600 nm. The ferric haem was then reduced by adding Na₂S₂O₄ to the protein solution, and the spectrum of the reduced enzyme was then recorded. Treatment of the Na₂S₂O₄-reduced sample with CO and comparison with the Na₂S₂O₄-reduced sample gave the CO-difference spectrum.

De-epoxypimaricin extraction and analysis

De-epoxypimaricin was extracted from the Δ PimD mutant *St. natalensis* 6D4 culture broths following published procedures [15]. Quantitative determination of pimaricin or de-epoxypimaricin was performed with a Waters 600 HPLC with a diode array ultraviolet detector set at 304 nm, fitted with a μ -Bondapak RP-C18 column (10 μ m; 3.9 mm \times 300 mm). Elution was with a gradient (1 ml/min) of 100% methanol (methanol concentration: 50%, 0–3 min; up to 90%, 3–12 min, 90%, 12–20 min; down to 50%, 20–25 min; 50%, 25–30 min). Retention time for pimaricin was 14.7 min, and for 4,5-de-epoxypimaricin it was 16.5 min.

Conversion of de-epoxypimaricin into pimaricin *in vitro*

A standard conversion of 4,5-de-epoxypimaricin into pimaricin was accomplished by combining 10–150 nM PimD, 10–250 μ M de-epoxypimaricin, 100 μ g/ml spinach ferredoxin, 0.2 unit/ml spinach ferredoxin-NADP⁺ reductase, 1.4 mM NADPH, 10 mM glucose 6-phosphate and 8 units/ml glucose-6-phosphate dehydrogenase in 50 mM Tris/HCl, pH 7.5. The reactions were carried out for different time periods at 30 °C and terminated by the addition of 8 vol. of butanol. The resulting organic extracts were dried and resuspended in methanol for HPLC analysis. The identities of de-epoxypimaricin and pimaricin were determined by LC/MS (liquid chromatography/mass spectrometry) using an ES-TOF (electrospray-time of flight) apparatus (Micromass, Manchester, U.K.). A pimaricin solution (5 mg/ml methanol) was used for tuning. Analyses were run using the positive-ionization mode. Chromatography and column characteristics were as indicated above.

RESULTS

Heterologous expression of *pimD*

The involvement of the product of the gene *pimD* in the formation of the epoxide group of the pimaricin molecule has been suggested on the basis of gene inactivation experiments [15], but has not been proven *in vitro*. To analyse the epoxidation reaction catalysed by PimD, the *pimD* gene was overexpressed in *E. coli*, and the corresponding product was purified and functionally characterized. For expression, the *pimD* gene was placed under the

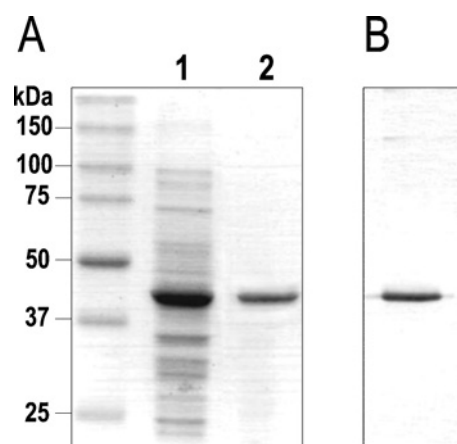


Figure 1 Purification of PimD in *E. coli* BL21

(A) SDS/PAGE (12% gels) of crude *E. coli* lysate expressing PimD (lane 1) and after purification by Ni²⁺ chromatography (lane 2). (B) Immunoblot detection of His₆-PimD with anti-His-tag antibodies.

control of a T5 promoter including a His₆-tag introduced at its 3' terminus (plasmid pMVM5; see the Experimental section). Induction of *E. coli* BL21(DE3)/pMVM5 with IPTG resulted in the production of a soluble 44 kDa protein as determined by SDS/PAGE (12% gels) analysis, consistent with the predicted molecular mass of PimD (43533 Da) (Figure 1). This band was absent from a control culture of BL21(DE3) containing the vector

plasmid pQE30 with no insert. Purification of His₆-PimD was achieved with a single chromatographic step on a Ni-NTA-agarose column. The purified His₆-PimD fraction contained a single detectable band on a Coomassie-Blue-stained SDS/12% polyacrylamide gel, and reacted strongly with anti-His-tag antibodies in Western blots (Figure 1).

PimD is an authentic cytochrome P450

The translated amino acid sequence of PimD was compared with those of other macrolide P450 enzymes. As expected, PimD contains the conserved signature sequences associated with cytochrome P450 enzymes, namely an O₂-binding site and a C-terminal haem-binding domain containing the invariant cysteine residue that co-ordinates the haem (Figure 2A).

The UV-visible absorption spectrum of the purified protein contained the characteristic 390–450 nm cytochrome P450 peak with a maximum at 415.5 nm. Reduction with Na₂S₂O₄ and exposure to CO gave the distinctive CO complex spectrum characteristic of cytochromes P450, with a maximum at 450 nm (Figure 2B). This spectral characterization provided the first physical evidence that PimD was in fact a cytochrome P450.

Conversion of de-epoxypimaricin into pimaricin *in vitro*

The enzymic activity of PimD was measured by converting its natural substrate 4,5-de-epoxypimaricin (isolated from the ΔPimD mutant *St. natalensis* 6D4, which accumulates de-epoxypimaricin; [15]) into pimaricin. Purified PimD protein was incubated at 30 °C with de-epoxypimaricin in the presence of spinach ferredoxin, spinach ferredoxin-NADP⁺ reductase and

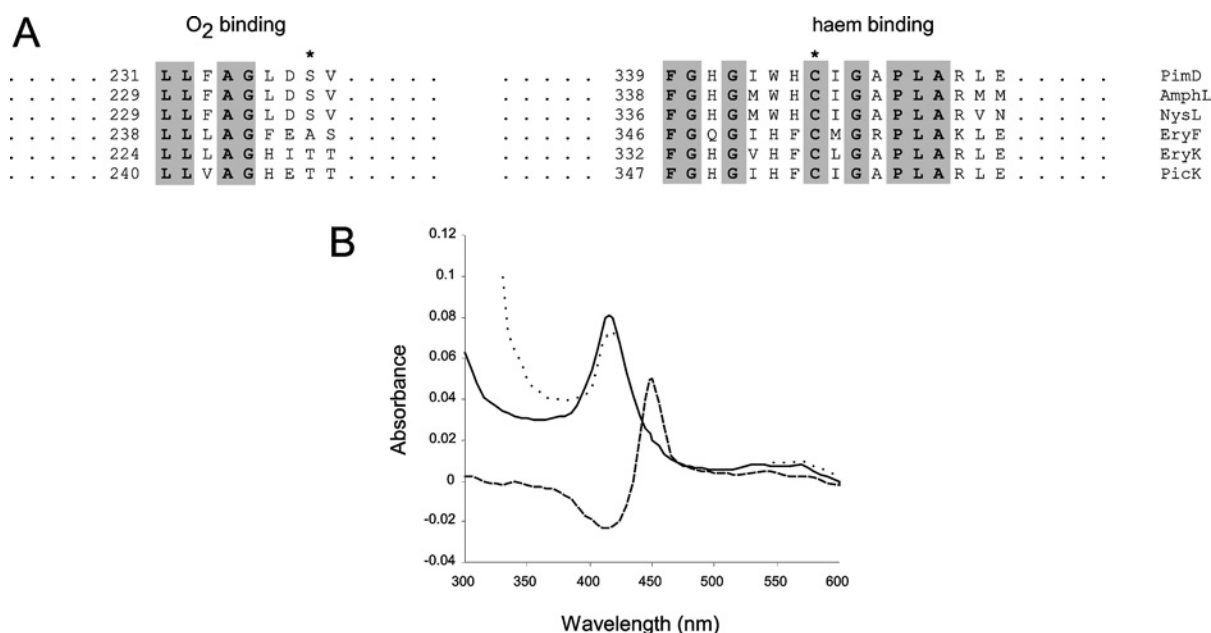


Figure 2 Identification of PimD as an authentic cytochrome P450

(A) Comparison of O₂-binding pockets and haem-binding sequences from some macrolide P450 mono-oxygenases. The asterisk on the left alignment shows the conserved threonine/serine (alanine in EryF) residues that are believed to be involved in O₂ scission, whereas the asterisk on the right indicates the haem-binding cysteine residue. Conserved amino acids are shown in bold and are highlighted. Numbers indicate amino acid residues from the N-terminus of the protein. AmphL, *St. nodosus* P450 involved in amphotericin biosynthesis (GenBank[®] accession number AAK73504); NysL, *St. noursei* P450 involved in nystatin biosynthesis (GenBank[®] accession number AAF71769); EryF (GenBank[®] accession number Q00441) and EryK (GenBank[®] accession number P48635), P450s from *Sa. erythraea* involved in erythromycin biosynthesis; PicK, methymycin/picromycin hydroxylase from *St. venezuelae* (GenBank[®] accession number AAC64105). (B) CO difference spectrum of purified PimD. The continuous line indicates the absorbance spectrum of the pure protein in storage buffer. The dotted line denotes the spectrum of the protein reduced with Na₂S₂O₄. The reduced CO-difference spectrum is indicated by a broken line.

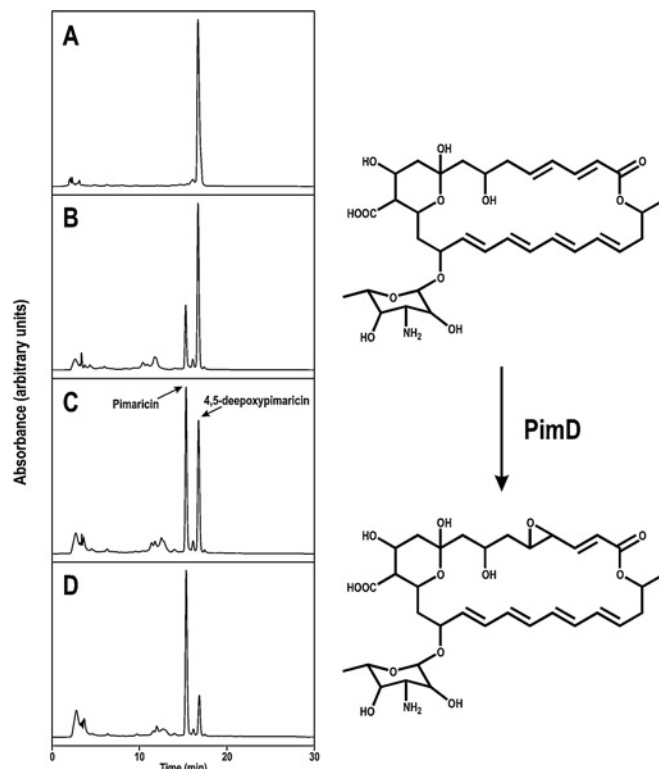


Figure 3 *In vitro* conversion of de-epoxypimaricin into pimarinic acid by PimD

The results of analytical HPLC after reaction with different enzyme concentrations are shown as follows: (A), no enzyme; (B) 25 nM PimD; (C) 50 nM PimD; (D) 100 nM PimD. Reactions were carried out for 30 min. Chromatographic peaks corresponding to 4,5-de-epoxypimaricin and pimarinic acid are indicated by arrows.

NADPH *in vitro*. An NADPH-regenerating system based on glucose 6-phosphate and glucose-6-phosphate dehydrogenase was also included in the reaction mixture. In the presence of such an electron-donating system, the enzyme readily converted de-epoxypimaricin into pimarinic acid. Figure 3 shows an enzyme-dependent conversion of de-epoxypimaricin into pimarinic acid, as measured by HPLC. The identity of the pimarinic acid peak was assessed by LC/MS. In the absence of the NADPH-regenerating system, no conversion was observed at all. Furthermore, in its presence, removal of any of the three remaining protein components of the reaction, namely the spinach ferredoxin, the ferredoxin-NADP⁺ reductase, or the PimD enzyme, resulted in the complete loss of catalytic activity (from a rate of 30.95 nmol of pimarinic acid formed/nmol of PimD per min when all components were present).

Optimal conditions for activity

In a standard 45 min reaction, the optimal temperature was found to be in the range 28–32 °C. Outside this range, the enzyme activity was lost rapidly, especially at higher temperatures. This activity loss was particularly evident at 37 °C, where only 30% activity remains after 15 min of incubation. Within the optimal temperature range, the linear phase of reaction lasted for 20 min, and the enzyme remained active for at least 2 h.

The pH-dependence of enzyme activity was measured in 50 mM Mes/NaOH, pH 5.5–7.5, 50 mM Tris/HCl, pH 7.0–8.5, and 50 mM glycine/NaOH, pH 8.5–9.5 buffers. The enzyme activity became apparent throughout the pH range 6.5–9.0, with a peak at pH 7.5 (results not shown).

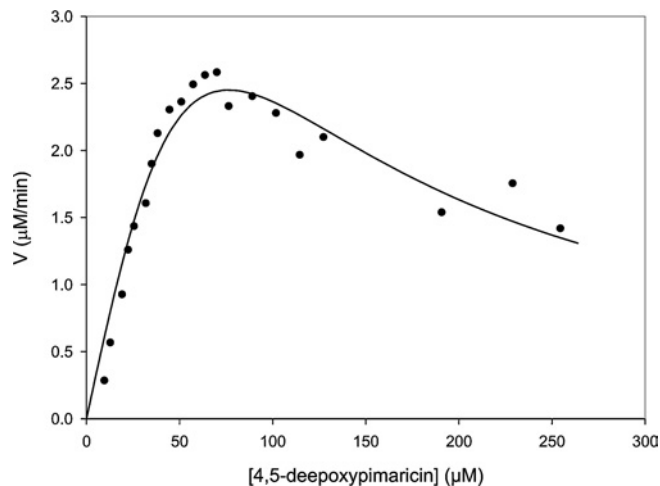


Figure 4 Least-squares fit of initial rate data

Initial rates were determined by applying a quadratic fit to the linear region of the time trace. The data were then fitted to the equation $v = V_{\max}/\{1 + (K_m/[S]) + ([S]/K_i)\}$.

Kinetic parameters of the reaction catalysed by His₆-PimD

Initial velocities for the epoxidation of 4,5-de-epoxypimaricin were determined by directly measuring the formation of pimarinic acid by HPLC. At a substrate concentration of 1 mM, the initial rate of epoxidation was found to correlate linearly with enzyme concentrations in the range 10–100 nM His₆-PimD. An enzyme concentration of 75 nM was selected to obtain steady-state kinetic parameters. Initial velocities were obtained with de-epoxypimaricin concentrations between 10 and 250 μM. Product formation was linear for the period monitored (0–20 min). A double-reciprocal plot of the data obtained indicated an asymptotic rise in 1/V at low values of 1/[S]. This behaviour is typical of substrate inhibition. For this reason, we fitted the initial velocity data to the equation $v = V_{\max}/\{1 + (K_m/[S]) + ([S]/K_i)\}$, which takes substrate inhibition into account (Figure 4). In this case, the steady-state parameters were a K_m of $93 \pm 29 \mu\text{M}$ and a V_{\max} of $6.8 \pm 1.5 \mu\text{M}/\text{min}$, corresponding to a k_{cat} value of $1.5 \pm 0.34 \text{ s}^{-1}$. Alternatively, at low substrate concentrations, where inhibition is negligible, the data could be fitted to the Michaelis–Menten equation, resulting in values for K_m of $33 \pm 5.5 \mu\text{M}$ and V_{\max} of $3.5 \pm 0.2 \mu\text{M}/\text{min}$. Assuming that all the enzyme is active, this would correspond to a k_{cat} value of $0.78 \pm 0.04 \text{ s}^{-1}$ and a k_{cat}/K_m of $0.024 \pm 0.003 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ (Table 1).

DISCUSSION

PimD [CYP161A2, according to the standard nomenclature (see <http://drnelson.utm.edu/CytochromeP450.html>)] represents the first polyene macrolide P450 mono-oxygenase to be expressed heterologously and studied, and constitutes the first oxidase tailoring a large-size macrolactone ring to be characterized in some detail. Thus far, only three macrolide hydroxylases have been studied regarding their kinetics *in vitro*. These are EryF and EryK, both involved in the hydroxylation of erythromycin A precursors, and PicK, involved in the oxidation of methymycin, neomethymycin and picromycin precursors (namely YC-17 and narbomycin). All of them catalyse hydroxylation of 12- and 14-membered macrolactones [7,16–18], while PimD uses a 26-membered aglycone as substrate.

Table 1 Steady-state kinetic parameters for His₆-PimD and other macrolide oxidases

Enzyme	Substrate	k_{cat} (s ⁻¹)	K_m (μM)	K_{cat}/K_m (μM ⁻¹ · s ⁻¹)	Source
PimD	4,5-De-epoxypimaricin	1.5 ± 0.34	93 ± 29	0.016 ± 0.002	This work
PimD*	4,5-De-epoxypimaricin	0.78 ± 0.04	33 ± 5.5	0.024 ± 0.003	This work
EryF	6-Deoxyerythronolide B	1.7	1.98 ± 0.26	0.85 ± 0.11	[16]
EryK	Erythromycin D	6.2 ± 1.2	44 ± 10	0.14 ± 0.042	[17]
EryK*	Erythromycin D	1.8 ± 0.18	8 ± 2.3	0.22 ± 0.068	[17]
PicK	YC-17	1.1 ± 0.08	130 ± 20	0.008 ± 0.004	[18]
PicK	Narbomycin	2.0 ± 0.3	88 ± 24	0.023 ± 0.01	[18]
PicK*	YC-17	0.6 ± 0.05	20.4 ± 5.6	0.029 ± 0.008	[7]
PicK*	Narbomycin	1.0 ± 0.11	43.7 ± 5.9	0.023 ± 0.018	[7]

* Apparent values obtained from fitting the Michaelis–Menten equation to the low-concentration data in the linear region of the saturation curve.

PimD tailors 4,5-de-epoxypimaricin, the immediate precursor of pimaricin [15], while it seems unable to use 4,5-de-epoxy-pimaricinolide as substrate. Mutants in the mycosaminyl transferase gene, *pimK*, present in the pimaricin gene cluster in *St. natalensis*, accumulate 4,5-de-epoxypimaricinolide as a major product (M. V. Mendes, J. F. Martín and J. F. Aparicio, unpublished work), indicating that epoxidation is impaired in the absence of glycosylation and suggesting that the oxidase shows strict molecular recognition of the glycosylated polyene substrate. Similar results have been observed upon inactivation of the *amphDIII* gene, which encodes a GDP-D-mannose 4,6-dehydratase that is involved in the biosynthesis of mycosamine, from the amphotericin gene cluster in *Streptomyces nodosus* [24]. In this case, the mutant accumulated 8-deoxyamphoteronolides, suggesting that glycosylation precedes C-8 hydroxylation, and that the AmphL mono-oxygenase that is responsible for the hydroxylation of 8-deoxyamphotericins at C-8 shows strict substrate specificity [24]. PimD and AmphL, and also their counterpart NysL from nystatin-producing *Streptomyces noursei* [11], belong to the same phylogenetic group, and are thought to be responsible of specific oxidations in the polyol region of polyene macrolide precursors [19].

It is unknown if the His₆-tag addition to the N-terminus of the protein affects the measured kinetic values of PimD. In any case, the k_{cat} value measured for His₆-PimD (0.8–1.5 s⁻¹, depending on the method used to calculate the kinetic parameters; see the Results section) is similar to the values reported for EryF towards its substrate 6-deoxyerythronolide B, EryK towards erythromycin D, and PicK towards both YC-17 and narbomycin (Table 1); while the K_m value (33–93 μM) is higher than those reported for EryF and EryK, and similar to the parameters for PicK (Table 1). This represents a substantially lower substrate specificity of PimD compared with that of EryF ($k_{cat}/K_m = 0.85 \mu\text{M}^{-1} \cdot \text{s}^{-1}$) and EryK ($k_{cat}/K_m = 0.14\text{--}0.22 \mu\text{M}^{-1} \cdot \text{s}^{-1}$), almost all of which can be attributed to the lower affinity of PimD towards its substrate, and similar to that of PicK towards its substrates, narbomycin and YC-17.

Interestingly, PimD was inhibited by a high concentration of its natural substrate de-epoxypimaricin. Substrate inhibition has previously been observed in the hydroxylations catalysed by EryK (towards erythromycin D) [17] and PicK (towards both YC-17 and narbomycin) [18].

Combinatorial biosynthesis is leading to a growing number of novel macrolide analogues. In many cases, however, the modified compounds lack biological activity, due to their deficiency in post-polyketide synthase oxidations, possibly due to the apparent strict substrate specificities of many of these oxidizing enzymes. The identification of novel macrolide mono-oxygenases with activities towards alternative substrates is therefore a crucial aspect

for the oxidative tailoring of novel polyketides, and should facilitate the search for new bioactive macrolide compounds. It is conceivable that PimD could also accept other substrates and prove to be valuable for the introduction of epoxy substituents into designer macrolides, including pimaricin, amphotericin or nystatin derivatives. The ease of its purification can greatly facilitate its use for the *in vitro* epoxidation of non-natural substrates that may require high concentrations of enzyme.

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REFERENCES

- Munro, A. W. and Lindsay, J. G. (1996) Bacterial cytochromes P-450. *Mol. Microbiol.* **20**, 1115–1125
- Haydock, S. F., Dowson, J. A., Dhillon, N., Roberts, G. A., Cortés, J. and Leadlay, P. F. (1991) Cloning and sequencing analysis of genes involved in erythromycin biosynthesis in *Saccharopolyspora erythraea*: sequence similarities between EryG and a family of S-adenosylmethionine-dependent methyltransferases. *Mol. Gen. Genet.* **230**, 120–128
- Stassi, D., Donadio, S., Staver, M. J. and Katz, L. (1993) Identification of a *Saccharopolyspora erythraea* gene required for the final hydroxylation step in erythromycin biosynthesis. *J. Bacteriol.* **175**, 182–189
- Merson-Davies, L. A. and Cundliffe, E. (1994) Analysis of five tylosin biosynthetic genes from the *tylBA* region of *Streptomyces fradiae* genome. *Mol. Microbiol.* **13**, 349–355
- Rodríguez, A. M., Olano, C., Méndez, C., Hutchinson, C. R. and Salas, J. A. (1995) A cytochrome P450-like gene possibly involved in oleandomycin biosynthesis by *Streptomyces antibioticus*. *FEMS Microbiol. Lett.* **127**, 117–120
- Betlach, M. C., Kealey, J. T., Betlach, M. C., Ashley, G. W. and McDaniel, R. (1998) Characterisation of the macrolide P-450 hydroxylase from *Streptomyces venezuelae* which converts narbomycin to picromycin. *Biochemistry* **37**, 14937–14942
- Xue, Y., Wilson, D., Zhao, L., Liu, H. and Sherman, D. H. (1998) Hydroxylation of macrolactones YC-17 and narbomycin is mediated by the *pikC*-encoded cytochrome P450 in *Streptomyces venezuelae*. *Chem. Biol.* **5**, 661–667
- Tang, L., Shah, S., Chung, L., Carney, J., Katz, L., Khosla, C. and Julien, B. (2000) Cloning and heterologous expression of the epothilone gene cluster. *Science* **287**, 640–642
- Molnár, I., Schupp, T., Ono, M., Zirkle, R. E., Milnamow, M., Nowak-Thompson, B., Engel, N., Toupet, C., Stratmann, A., Cyr, D. D. et al. (2000) The biosynthetic gene cluster for the microtubule-stabilising agents epothilones A and B from *Sorangium cellulosum* So ce90. *Chem. Biol.* **7**, 97–109
- Caffrey, P., Lynch, S., Flood, E., Finnan, S. and O'Liainy, M. (2001) Amphotericin biosynthesis in *Streptomyces nodosus*: deductions from analysis of polyketide synthase and late genes. *Chem. Biol.* **8**, 713–723
- Brautaset, T., Sekurova, O. N., Sletta, H., Ellingsen, T. E., Strom, A. R., Valla, S. and Zotchev, S. B. (2000) Biosynthesis of the polyene antifungal antibiotic nystatin in *Streptomyces noursei* ATCC 11455: analysis of the gene cluster and deduction of the biosynthetic pathway. *Chem. Biol.* **7**, 395–403

- 12 Aparicio, J. F., Colina, A. J., Ceballos, E. and Martín, J. F. (1999) The biosynthetic gene cluster for the 26-membered ring polyene macrolide pimaricin. *J. Biol. Chem.* **274**, 10133–10139
- 13 Aparicio, J. F., Fouces, R., Mendes, M. V., Olivera, N. and Martín, J. F. (2000) A complex multienzyme system encoded by five polyketide synthase genes is involved in the biosynthesis of the 26-membered polyene macrolide pimaricin in *Streptomyces natalensis*. *Chem. Biol.* **7**, 895–905
- 14 Molnár, I., Aparicio, J. F., Haydock, S. F., Khaw, L. E., Schwecke, T., König, A., Staunton, J. and Leadlay, P. F. (1996) Organisation of the biosynthetic gene cluster for rapamycin in *Streptomyces hygroscopicus*: analysis of genes flanking the polyketide synthase. *Gene* **169**, 1–7
- 15 Mendes, M. V., Recio, E., Fouces, R., Luiten, R., Martín, J. F. and Aparicio, J. F. (2001) Engineered biosynthesis of novel polyenes: a pimaricin derivative produced by targeted gene disruption in *Streptomyces natalensis*. *Chem. Biol.* **8**, 635–644
- 16 Andersen, J. F., Tatsuta, K., Gunji, H., Ishiyama, T. and Hutchinson, C. R. (1993) Substrate specificity of 6-deoxyerythronolide B hydroxylase, a bacterial cytochrome P450 of erythromycin biosynthesis. *Biochemistry* **32**, 1905–1913
- 17 Lambalot, R. H., Cane, D. E., Aparicio, J. J. and Katz, L. (1995) Overproduction and characterization of the erythromycin C-12 hydroxylase, EryK. *Biochemistry* **34**, 1858–1866
- 18 Graziani, E. I., Cane, D. E., Betlach, M. C., Kealey, J. T. and McDaniel, R. (1998) Macrolide biosynthesis: a single cytochrome P450, PicK, is responsible for the hydroxylations that generate methymycin, neomethymycin, and picromycin in *Streptomyces venezuelae*. *Bioorg. Med. Chem. Lett.* **8**, 3117–3120
- 19 Aparicio, J. F., Caffrey, P., Gil, J. A. and Zotchev, S. B. (2003) Polyene antibiotic biosynthesis gene clusters. *Appl. Microbiol. Biotechnol.* **61**, 179–188
- 20 Antón, N., Mendes, M. V., Martín, J. F. and Aparicio, J. F. (2004) Identification of PimR as a positive regulator of pimaricin biosynthesis in *Streptomyces natalensis*. *J. Bacteriol.* **186**, 2567–2575
- 21 Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F. and Hopwood, D. A. (2000) *Practical Streptomyces Genetics*, John Innes Foundation, Norwich
- 22 Sambrook, J. and Russell, D. W. (2001) *Molecular Cloning: a Laboratory Manual*, 3rd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- 23 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–252
- 24 Byrne, B., Carmody, M., Gibson, E., Rawlings, B. and Caffrey, P. (2003) Biosynthesis of deoxyamphotericins and deoxyamphoterololides by engineered strains of *Streptomyces nodosus*. *Chem. Biol.* **10**, 1215–1224

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