Hydroxylation of macrolactones YC-17 and narbomycin is mediated by the *pikC*-encoded cytochrome P450 in *Streptomyces venezuelae*

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Background: *Streptomyces venezuelae* produces two groups of antibiotics that include the 12-membered ring macrolides methymycin and neomethymycin, and the 14-membered ring macrolide pikromycin. Methymycin and pikromycin are derived from the corresponding precursors, YC-17 and narbomycin, respectively, by hydroxylation of the tertiary carbon position (C-10 in YC-17 or C-12 in narbomycin) on the macrolactone ring. In contrast, neomethymycin is derived from YC-17 by hydroxylation of the secondary carbon (C-12) of the propionyl starter unit sidechain.

Results: Using a genetic and biochemical approach we have characterized a single P450 hydroxylase (PikC) in the methymycin/pikromycin biosynthetic gene cluster (*pik*) from *S. venezuelae*. Inactivation of *pikC* abolished production of all hydroxylated macrolides, with corresponding accumulation of YC-17 and narbomycin in the culture medium. The enzyme was produced efficiently and purified as a His-tagged protein from recombinant *Escherichia coli* cells. Purified PikC effectively converts YC-17 into methymycin and neomethymycin and narbomycin into pikromycin *in vitro*.

Conclusions: These results demonstrate that PikC is responsible for the conversion of YC-17 to methymycin and neomethymycin, and narbomycin to pikromycin in *S. venezuelae*. This substrate flexibility is unique and represents the first example of a P450 hydroxylase that can accept 12- and 14-membered ring macrolides as substrates, as well as functionalize at two positions on the macrolactone system. The broad substrate specificity of PikC provides a potentially valuable entry into the construction of novel macrolide- and ketolide-based antibiotics.

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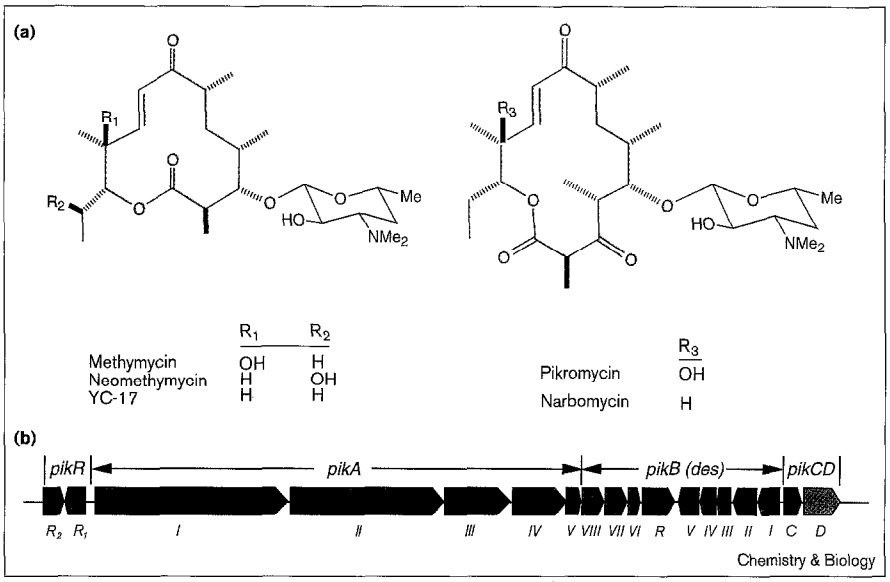
Introduction

Cytochromes P450 are one of the most widely distributed family of enzymes in nature, catalyzing the oxidation of a broad range of physiological and nonphysiological compounds [1]. Although hundreds of P450 hydroxylases have been examined in the oxidative metabolism of xenobiotics and steroids, only a small number have been studied in bacterial secondary metabolism, especially in macrolide antibiotic biosynthetic pathways of actinomycetes. Of the latter, two enzymes, EryF [2,3] and EryK [4] have been purified and the EryF structure has been determined by X-ray crystallography [5]. Both enzymes are involved in the biosynthesis of erythromycin A in *Saccharopolyspora erythraea*, and each is responsible for the hydroxylation at a single position on the erythromycin aglycone. Specifically, EryF is 6-deoxyerythronolide B (6-DEB) 6-hydroxylase, and EryK is erythromycin D (ErD) 12-hydroxylase. As prototype P450 hyroxylases involved in secondary metabolism, EryF and EryK are considered to exhibit strict substrate specificity. This characteristic extends to similar enzymes in other macrolide pathways. For example, rapamycin biosynthesis requires hydroxylation at two positions and two P450 hydroxylase genes have been identified in the *rap* biosynthetic cluster [6,7]. To date, there has been only one report describing a P450 hydroxylase that can function at two positions on a macrolactone substrate [8].

Streptomyces venezuelae is known to produce methymycin, neomethymycin and pikromycin, three macrolide antibiotics derived from two macrolactone ring systems. Methymycin and neomethymycin are derived from the 12-membered ring macrolide YC-17 by hydroxylation at the C-10 and C-12 positions, respectively. Pikromycin is derived from the 14-membered ring ketolide narbomycin by hydroxylation at C-12 (equivalent to C-10 in YC-17, Figure 1a). A previous analysis of P450 hydroxylase activity in cell-free extracts of *S. venezuelae* suggested that the bacterium might utilize two distinct P450 activities to convert

Figure 1



(a) The structure of methymycin, neomethymyin, pikromycin and related compounds. **(b)** Organization of the methymycin/pikromycin biosynthetic gene cluster (*pik*) in *S. venezuelae* ATCC15439. Each arrow represents an open reading frame (ORF). Purple, resistance genes; blue, polyketide synthase genes; red, desosamine biosynthetic genes; green, cytochrome P450 hydroxylase and its transcriptional activator (putative) genes.

YC-17 into methymycin and neomethymycin [9]. Recent characterization of the methymycin/pikromycin biosynthetic pathway in *S. venezuelae* revealed a single P450 hydroxylase gene (*pikC*), however [10]. Here we provide evidence that the corresponding protein (PikC) is responsible for hydroxylation at both the C-10 and C-12 positions, thereby producing methymycin and neomethymycin. We also show that PikC catalyzes the conversion of 14-membered ring ketolide narbomycin to pikromycin.

Results

Nucleotide sequencing of the *pikCD* operon

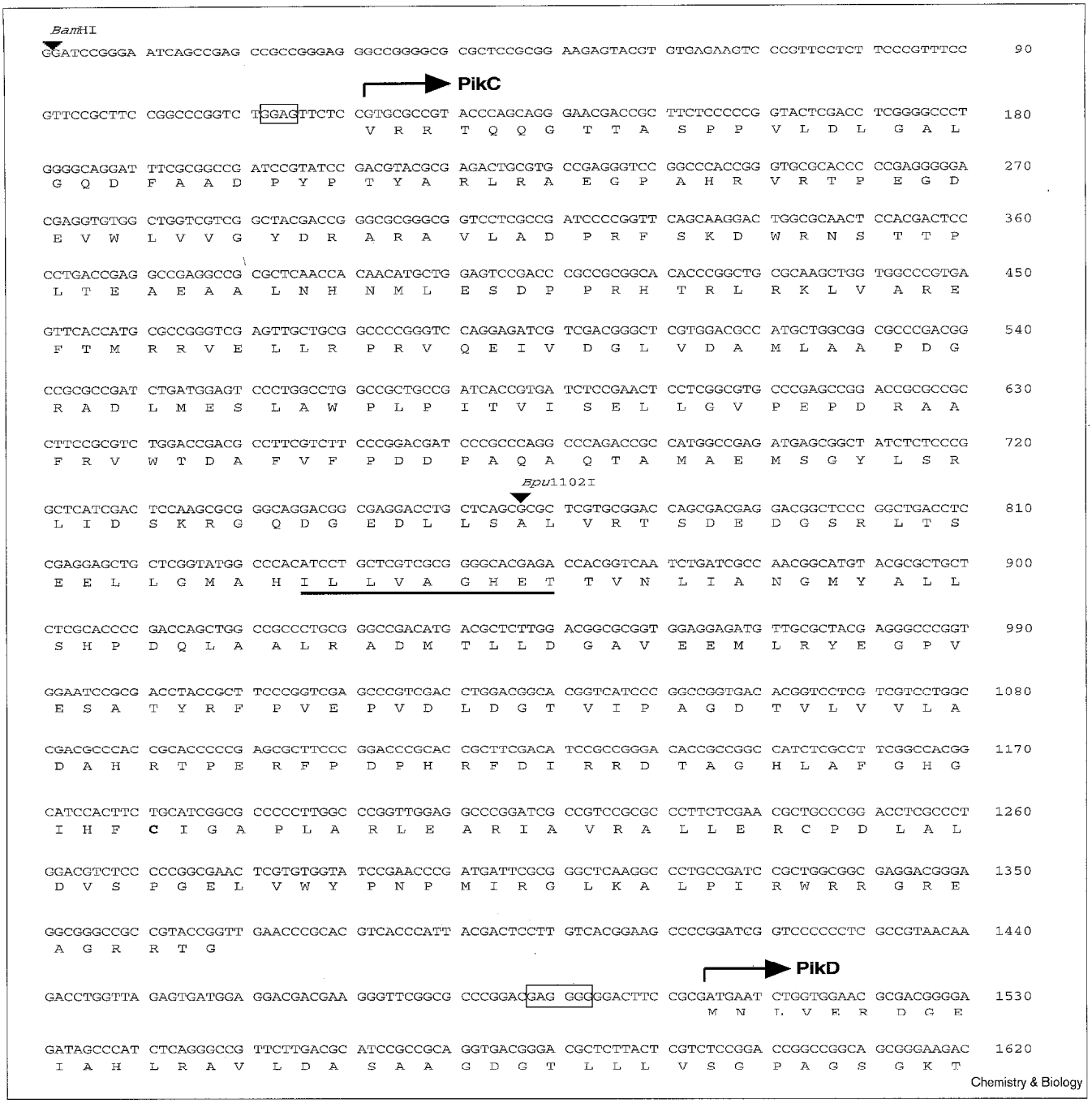
The entire gene cluster for methymycin, neomethymycin and pikromycin biosynthesis (*pik*) in *S. venezuelae* was identified using a heterologous hybridization approach [10]. The *pik* cluster (Figure 1b) contains a resistance locus, *pikR*, and three biosynthetic loci including the polyketide synthase (PKS) genes (*pikAI–AV*), the desosamine biosynthetic genes (*des*) and a P450 hydroxylase gene encoded by *pikC* [10]. Recently, *pikA* and *des* have been shown to mediate the biosynthesis of the macrolactone and desosamine groups in both YC-17 and narbomycin [10]. Immediately downstream of the *pikA* and *des* genes is the *pikCD* locus (Figure 1b). Its complete nucleotide sequence (approximately 5 kilobases) was determined and two open reading frames were identified. PikC encodes a peptide of 416 amino acids (molecular weight 46 kDa), and belongs to the superfamily of cytochrome P450 monooxygenases. It has a heme-binding motif centered at Cys354 that is proposed to function as the heme iron ligand [4,5]. A region of sequence established previously as the dioxygen-binding motif [4] is located at amino-acid positions 239–247 with the Thr247 OH group presumably forming a hydrogen bond with the heme iron (Figure 2).

Amino-acid sequence comparison of PikC revealed strong similarity to other cytochrome P450 hydroxylases, especially those identified from polyketide biosynthetic pathways. Strongest similarity is with the predicted product of *orf405* [3] and the EryF protein [3] in *S. erythrea*. Although *orf405* encodes a P450 of unknown function, EryF is responsible for the C-6 hydroxylation in erythromycin biosynthesis [11]. PikC also shows very high similarity to a P450 involved in carbomycin biosynthesis [12], MycG [8] involved in mycinamicin biosynthesis, RapN [6,7] involved in rapamycin biosynthesis, and OleP [13] involved in oleandomycin biosynthesis. Each of these P450s is predicted to be active at a specific position on a macrolactone ring, similar to EryF and EryK, except MycG; the MycG cytochrome P450 catalyzes both hydroxylation and epoxidation at two distinct positions on its natural substrate, mycinamicin IV [8].

Surprisingly, sequence comparison revealed that PikC is more similar to EryF than to EryK (54% versus 30% similarity). Because one of the reactions catalyzed by PikC is narbomycin (C-12) hydroxylation (see below), we expected greater similarity between PikC and EryK (ErD 12-hydroxylase) than to EryF (6-DEB 6-hydroxylase). The fact that PikC is more similar to EryF in sequence could indicate that the primary sequence of P450 hydroxylases is not necessarily a good predictor of substrate specificity.

Just downstream of *pikC* is *pikD*, which encodes a predicted 101 kDa (928 amino acid) protein. The entire pikD sequence shows high similarity to the putative transcriptional activator in a second PKS cluster from rapamycin-producing *Streptomyces hygroscopicus* [14]. PikD is also very similar to ORF H in the rapamycin biosynthetic cluster [7], and to a regulatory protein in the cholesterol

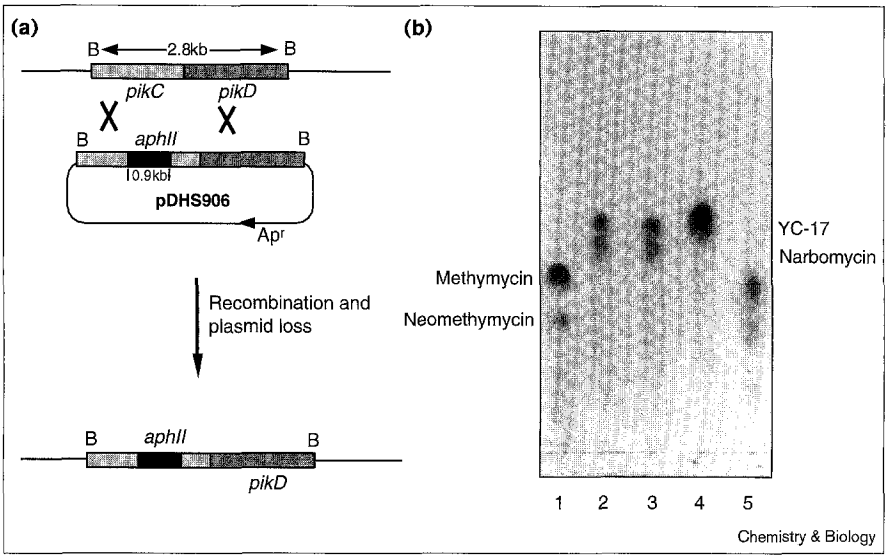
Figure 2



The nucleotide and deduced amino-acid sequence of *pikC*. The ordinate corresponds to the nucleotide sequence, and the *pikD* sequence was arbitrarily truncated. (The sequence for *pikCD* was deposited in GenBank under the accession number AF079139). The *pikC*-coding region starts at nucleotide 122 with a putative ribosome binding site (boxed nucleotides) upstream of the GTG start codon . The heme-binding cysteine residue (Cys354) is in bold, and the dioxygen-binding pocket amino acids (239–247) are underlined. Inactivation of *pikC* was conducted by inserting a kanamycin resistance gene, *aphII*, at the *Bpu*1102I restriction site (see text for details).

oxidase-cytochrome P450 operon from *Streptomyces* sp. SA-COO [15]. The carboxy-terminal half of PikD shows high sequence similarity to a transcriptional activator in the amino hydroxybenzoic acid (AHBA) biosynthetic pathway in *Actinosynnema pretiosum* [16], as well as significant similarity to the DNA-binding protein (regulatory component) in bacterial two-component systems that play a key role in environmental sensing [17]. We propose, therefore, that PikD is an activator that could specifically regulate expression of the *pikCD* operon; a more general regulatory

Figure 3



(a) Schematic illustration of *pikC* inactivation by homologous recombination. Abbreviations: B, *Bam*HI; Ap^r, apramycin resistance; *aphII*, kanamycin resistance gene. **(b)** Thin-layer chromotography (TLC) analysis of antibiotic production in the *pikC* disruption mutant strains. Lane 1, methymycin and neomethymycin standard; lane 2, mutant AX906-11; lane 3, mutant AX906-12; lane 4, YC-17 standard compound; and lane 5, wild-type *S. venezuelae*.

role for the entire *pik* cluster is also possible, however. A number of genes involved in primary metabolic functions for *S. venezuelae* were found immediately downstream of *pikCD*. We believe that this *pikCD* locus represents the right-hand limit of the *pik* biosynthetic gene cluster [10].

Mutational analysis of *pikC*

To establish whether *pikC* encodes the enzyme responsible for hydroxylation of both YC-17 and narbomycin, the gene was disrupted and the resulting mutant strain analyzed for macrolide antibiotic production. PikC was inactivated by inserting a kanamycin resistance gene (*aphII*) within the *pikC*-coding region by homologous recombination between the chromsome and the disruption plasmid pDHS906 (Figure 3a). Two double-crossover mutant strains (AX906-11 and AX906-12) were obtained. Southern blot analysis indicated that they are genotypically identical, with the *aphII* gene correctly inserted into the chromosomal copy of *pikC* (data not shown). The AX906 mutant has normal morphology compared to wild-type *S. venezuelae*, indicating that the genetic disruption had no detrimental effect on bacterial growth. Upon extraction of macrolide products, *S. venezuelae* AX906 was found to have accumulated macrolactone precursors YC-17 (75 mg/l) and narbomycin (20 mg/l), and was completely devoid of the normal hydroxylated compounds, methymycin, neomethymycin and pikromycin (Figure 3b). This result provided strong evidence that the product of *pikC* is solely responsible for catalyzing the hydroxylation of YC-17 to methymycin and neomethymycin, as well as narbomycin to pikromycin in *S. venezuelae*.

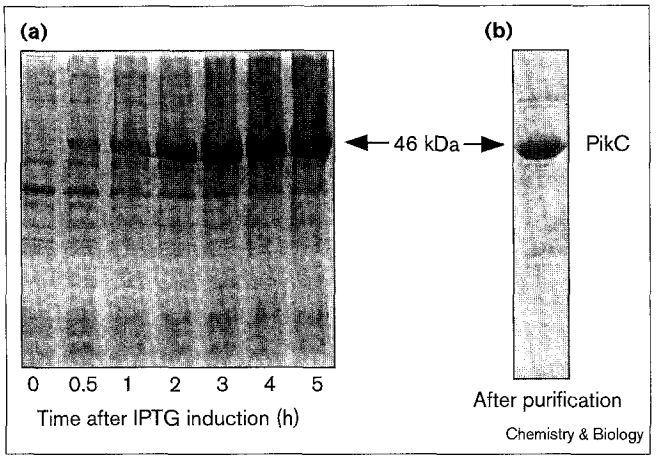
Heterologous expression of PikC

To analyze PikC in more detail, the *pikC* gene was overexpressed in *Escherichia coli* and the corresponding product purified and functionally characterized. The expression was placed under the control of a T7 promoter including a 6 × His-tag introduced at its amino terminus (see the Materials and methods section). After IPTG (isopropyl-β-D-thiogalactopyranoside) induction a protein corresponding to the size of PikC (46 kDa) was detected in recombinant *E. coli* BL21 cells (Figure 4a). Use of low concentrations of inducer (0.075 mM IPTG) and reduced culture temperatures (25°C) resulted in synthesis of soluble PikC protein by *E. coli* BL21. One-step purification was conducted using Ni-NTA agarose column to obtain the His-tagged protein to near homogeneity (Figure 4b). The purified product has a molecular weight of 46 kDa and reacted strongly with anti-His-tag antibody in Western blots (data not shown). UV absorption spectrum of the protein also established the identity of the purified protein as PikC cytochrome P450 hydroxylase.

Conversion of YC-17 and narbomycin to methymycin/neomethymycin and pikromycin *in vitro*

The enzymatic activity of PikC was measured by converting the natural substrates YC-17 and narbomycin (isolated from the AX906 mutant strain of *S. venezuelae*) to the corresponding products. When purified PikC was mixed with YC-17 at 30°C in the presence of spinach ferredoxin, spinach ferredoxin–NADP$^+$ reductase and NADPH *in vitro*, the enzyme readily converted YC-17 to methymycin and neomethymycin. In a similar reaction, PikC converted narbomycin into pikromycin. These results, together with the *pikC* disruption experiment, confirmed that PikC is capable of catalyzing the hydroxylations leading to methymycin/neomethymycin and pikromycin (Figure 5). Following the procedure developed for EryK [18], the steady-state kinetic parameters of the overall PikC hydroxylation reaction were

Figure 4



(a) Heterologous expression and **(b)** purification of PikC in *E. coli* BL21. The figure shows a Coomassie brilliant blue-stained SDS polyacrylamide gel, which represents protein expression after 0, 0.5, 1, 2, 3, 4 and 5 hours of IPTG induction. PikC after Ni-NTA column purification is shown in (b).

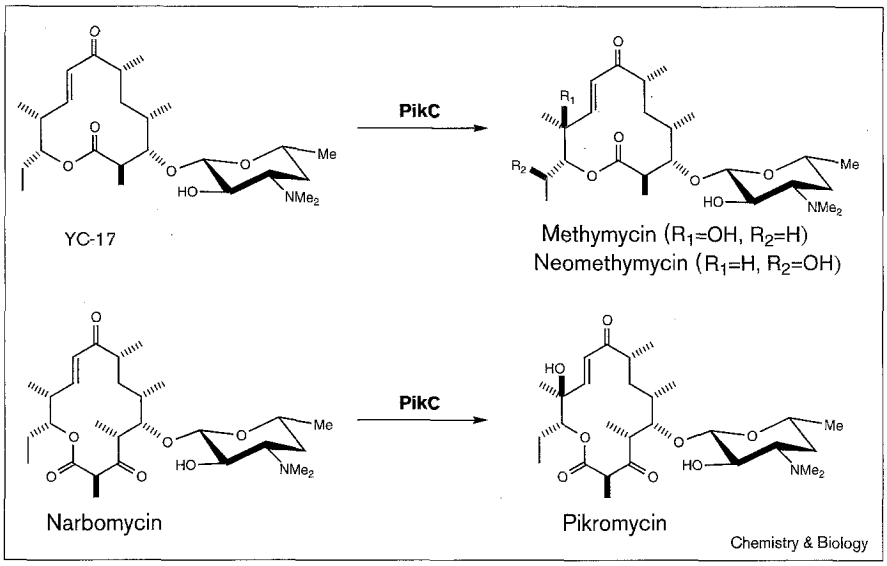
determined. PikC has a K_M of 20.4 ± 5.6 μM and a k_{cat} of 35.55 ± 2.9 min^{-1} for YC-17 hydroxylation, and a K_M of 43.7 ± 5.9 μM and a k_{cat} of 60.1 ± 6.5 min^{-1} for narbomycin conversion. The experimentally determined K_M values are very similar to those found for EryK towards ErD (its natural substrate), although the k_{cat} value for PikC is lower than for EryK. Interestingly, PikC activity was inhibited by a high concentration of YC-17 (a phenomenon also observed for EryK [18] (toward ErD) but not by narbomycin.

In *S. venezuelae* cell-free extracts Cane and Graziani [9] recently determined the hydroxylation reaction kinetics of methymycin and neomethymycin production from YC-17. Using purified PikC enzyme, however, we observed that bioconversion parameters are variable under different buffer conditions. Similarly, the observed product ratio between methymycin and neomethymycin in wild-type *S. venezuelae* also varies depending on specific production and cell-harvesting conditions. An analysis based on the overall hydroxylation reaction from substrate (YC-17 and narbomycin) therefore appears to be a more convenient and perhaps more reliable method for determining the kinetic parameters of PikC. We have recently become aware of work on hydroxylation of macrolactones similar to our own work [19].

Significance

Streptomyces venezuelae produces two groups of macrolide antibiotics; the 12-membered ring compounds methymycin and neomethymycin and the 14-membered ring ketolide pikromycin. YC-17 and narbomycin, the metabolic precursors of methymycin and pikromycin, are hydroxylated at the tertiary carbon positions of their macrolactone rings (C-10 in YC-17 or C-12 in narbomycin). These two different hydroxylation reactions were thought to be catalyzed by two different cytochrome P450 hydroxylases, because such hydroxylases identified to date exhibit strict substrate specificity. Sequence analysis of the gene cluster responsible for macrolide antibiotic synthesis in *S. venezuelae* revealed only one cytochrome P450 hydroxylase, so we surmised that this single enzyme (PikC) might be able to catalyze both reactions. This report includes the sequencing, genetic disruption and functional characterization of the *pikC* locus, including heterologous expression and purification of the PikC protein from *Escherichia coli* and biochemical study of

Figure 5



Conversion of YC-17 and narbomycin by purified PikC *in vitro*. See text for details of reaction conditions.

the PikC P450 hydroxylase *in vitro*. We show that PikC is the sole enzyme responsible for hydroxylation at the C-10 and C-12 positions of YC-17 to produce methymycin the neomethymycin, as well as the C-12 position of narbomycin to produce pikromycin in *S. venezuelae*. PikC is the first reported cytochrome P450 hydroxylase that both accepts macrolide substrates of different ring size and catalyzes addition of a hydroxyl group at two distinct positions on a macrolactone. This remarkable substrate and regiochemical flexibility could provide significant potential for production of novel macrolide and ketolide antibiotics through combinatorial-based and semi-synthetic strategies. The ready availability of PikC in soluble form provides an opportunity to investigate its substrate interactions and the basis of its unusual substrate recognition.

Materials and methods

Bacterial strains and media

E. coli DH5α was used throughout the study as a cloning host, and LB medium was used for *E. coli* propagation. *E. coli* BL21 DES (Novagen) was used as the expression host, and NZCYM [20] was the medium used for protein expression. *S. venezuelae* ATCC 15439 was obtained as a freeze-dried pellet from ATCC. Media for vegetative growth and antibiotic production were used as described previously [21]. Briefly, SGGP liquid medium was used for propagation of *S. venezuelae*. Sporulation agar (1 g yeast extract, 1 g beef extract, 2 g tryptose, trace amount of $FeSO_4$, 10 g glucose and 15 g agar in 1 l of dH_2O) was used for production of *S. venezuelae* spores. Methymycin production was conducted in either SCM or vegetative medium as described previously [21] and pikromycin production was performed in glucose-peptone medium [22].

Plasmids and DNA manipulation procedures

Plasmid pUC119 was used as the routine cloning vector, and pET-28(a) (Novagen) was the vector used to construct the PikC expression plasmid, pDHS616. Plasmid vectors for gene disruption were derived from pKC1139 [23], which is a conjugative shuttle plasmid for *E. coli* and *Streptomyces* sp. Plasmid, cosmid and genomic DNA preparation, restriction-enzyme digestion, fragment isolation and cloning were performed using standard procedures [20].

DNA sequencing and analysis

An exonuclease III (Exo III)-nested deletion combined with polymerase chain reaction (PCR)-based double-stranded DNA sequencing was employed to sequence the *pikCD* operon. First, the locus was subcloned as a 2.8 kb *Bam*HI fragment and a 3 kb *Bam*HI–*Sph*I fragment. Then the subclones were treated with Exo III to create a nested deletion series and sequenced using universal primers on the pUC119 vector. The Exo III procedure followed the Erase-a-Base protocol (Stratagene) and DNA sequencing reactions were performed using the Dye Primer Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The nucleotide sequences were read from an ABI PRISM 377 sequencer on both DNA strands. DNA and deduced protein sequence analyses were performed using GeneWorks or GCG sequence analysis packages (Oxford Molecular Group) employing the program-default specific parameters.

Gene disruption

A replicative plasmid-mediated homologous recombination approach was developed to conduct gene disruption in *S. venezuelae* [10]. Plasmids for insertional inactivation (pDHS906) were constructed by cloning a kanamycin-resistance gene, *aphII*, into a 2.8 kb *Bam*HI fragment into a *Bpu*1102I site (Figure 3a). Because the *Bpu*1102I site is upstream of the PikC P450 active-site sequences (Figure 2), the insertion should lead to complete inactivation of PikC activity. Disruption plasmid pDHS906 was introduced into *S. venezuelae* via RK4-mediated conjugation [23]. Spores from individual transconjugants were cultured on nonselective plates to induce recombination. The cycle was repeated three times to enhance the probability for recombination. Double crossovers yielding targeted gene disruption mutants were selected and screened for the kanamycin-resistant, apramycin-sensitive (Kan^R Apr^S) phenotype, and the mutant genotype was confirmed by Southern blot hybridization of genomic DNA.

Antibiotic extraction and analysis

Methymycin, neomethymycin, narbomycin and pikromycin were extracted following published procedures [24]. TLC was routinely used to detect YC-17, methymycin, neomethymycin, narbomycin and pikromycin. The solvent system for TLC was chloroform : methanol : 25% ammonium hydroxide (90:10:1) followed by vanillin staining (0.75% vanillin, 1.5% H_2SO_4 in methanol) and heating. Compounds were purified by flash column chromatography and high-performance liquid chromatography mass (HPLC), and analyzed further using ^1H, ^{13}C nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry.

Construction of pDHS616 for heterologous expression

PikC was amplified for insertion into the His-tag expression vector pET-28(a) using PCR. The forward primer (5′-GGT CTG GAG TTC CAT ATG CGC CGT ACC CAG CAG GGA ACG-3′) contained a unique *Nde*I site that replaced the native GTG translational start codon with ATG, the start codon contained within pET-28(a). The reverse primer (5′-GAG TAA GCT TGG GTG ACG TGC GGG TTC AAC CGG-3′) carried a *Hind*III site, as well as the TGA translational stop codon. The amplified *pikC* and pET-28(a) were sequentially digested with *Nde*I and *Hind*III before being ligated together creating pDHS616.

Expression and purification of soluble PikC

E. coli cells were grown at 37°C in 1.5 l NZCYM media containing 50 μg/ml of kanamycin until $OD_{600} = 1.0$. Expression of PikC was induced by adding IPTG to a final concentration of 0.075 mM, and reducing the incubation temperature to 25°C. The cells were allowed to grow overnight, after which they were harvested and suspended in 40 ml of lysis buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 10 mM imidazole). Lysis was accomplished on a Virsonic 475 (Virtis) sonicator using power level 5 in 45 s bursts followed by 15 s on ice. The insoluble material was separated by centrifugation at 15,000 rpm in a Beckman JA-20 rotor at 4°C. The soluble fraction was collected and incubated for 1 h at 0°C after addition of 10 ml Ni-NTA agarose (Qiagen). The slurry was added onto an empty column and the flow through collected for later analysis. The column was then washed with 100 ml of wash buffer (50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 20 mM imidazole) followed by collection after addition of 3×5 ml of elution buffer (100 mM KH_2PO_4 (pH 8.0), 300 mM NaCl, 250 mM imidazole). The elution fractions were combined and precipitated with 60% ammonium sulfate. Desalting was attained by dissolving the protein pellet in conversion buffer (50 mM NaH_2PO_4 (pH 7.3), 1 mM EDTA, 0.2 mM DTT, 10% glycerine) and passing through a Pharmacia PD-10 column.

Conversion of YC-17 and narbomycin in vitro

The initial conversion was accomplished by combining 1 μM desalted PikC, 0.5 mM narbomycin or YC-17, 3.5 μM spinach ferredoxin, 0.1 Units spinach ferredoxin–$NADP^+$ reductase, and 1mM NADPH in a total volume of 1 ml conversion buffer. The reaction was carried out for 10 min at 30°C and was terminated by extraction, using 3×1 ml of $CHCl_3$. The resulting organic extracts were combined, dried and resuspended in 100 μl methanol. Aliquots of 7 μl of the extracted compounds were used for TLC analysis.

Kinetic studies of PikC

The kinetics of PikC conversion of YC-17 or narbomycin were determined following the procedure developed for EryK [18]. Briefly, assuming a 1:1 stoichiometric relationship between NADPH consumption and substrate hydroxylation, initial velocities were measured spectrophotometrically at various substrate concentrations (5–500 μM)

by monitoring the rate of absorbance decay at 340 nm over time. Data was collected using a Shimadzu UV160U spectrophotometer in kinetics mode, acquiring at 340 nm, and equilibrated by placement in a 30°C warm room. The assay mixture contained 1 μM PikC, 9 μM spinach ferredoxin, 170 μM of NADPH, and 5–500 μM of substrate (YC-17 or narbomycin) suspended in 1 ml conversion buffer. The assay mixture was mixed in a 1 cm Hellma quartz cuvette and placed in the spectrophotometer. The reaction was initiated by adding of 0.1 U spinach ferredoxin–$NADP^+$ reductase and the rate of NADPH consumption was monitored over 100 s. The data from the first 20 s reaction were used to fit the Michaelis–Menten equation to determine the enzymatic kinetic parameters.

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