Characterization of the Macrolide P-450 Hydroxylase from *Streptomyces venezuelae* Which Converts Narbomycin to Picromycin[†,‡]

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ABSTRACT: The post-polyketide synthase (PKS) biosynthetic tailoring of macrolide antibiotics usually involves one or more oxidation reactions catalyzed by cytochrome P450 monooxygenases. As the specificities of members from this class of enzymes vary significantly among PKS gene clusters, the identification and study of new macrolide P450s are important to the growing field of combinatorial biosynthesis. We have isolated the cytochrome P450 gene *picK* from *Streptomyces venezuelae* which is responsible for the C-12 hydroxylation of narbomycin to picromycin. The gene was located by searching regions proximal to modular PKS genes with a probe for macrolide P450 monooxygenases. The overproduction of PicK with a C-terminal six-His affinity tag (PicK/6-His) in *Escherichia coli* aided the purification of the enzyme for kinetic analysis. PicK/6-His was shown to catalyze the in vitro C-12 hydroxylation of narbomycin with a k_{cat} of 1.4 s^{-1}, which is similar to the value reported for the related C-12 hydroxylation of erythromycin D by the EryK hydroxylase. The unique specificity of this enzyme should be useful for the modification of novel macrolide substrates similar to narbomycin, in particular, ketolides, a promising class of semisynthetic macrolides with activity against erythromycin-resistant pathogens.

Polyketide cytochrome P450 monooxygenases catalyze the site-specific oxidation of the precursors to many macrolide antibiotics, including erythromycin (*1*−*3*), tylosin (*4*), mycinamicin (*5*), oleandomycin (*6*), and methymycin (*7*). These reactions occur in the late stages of biosynthesis after formation of the macrocycle by the polyketide synthase (PKS).[1] The biological importance of the resulting hydroxyl and/or epoxide substituents to such compounds is often represented by a significant increase in antibiotic potency. Although cytochrome P450s are common in macrolide gene clusters, there are considerable variations in the substrates utilized and the positions of oxidation catalyzed by these enzymes. Thus far, the erythromycin hydroxylases EryF and EryK from *Saccharopolyspora erythraea* are the only enzymes from this family to be investigated in detail. Both enzymes catalyze hydroxylation of 14-carbon macrolactones; however, the substrate specificity of each enzyme is quite different. EryF catalyzes C-6 hydroxylation of the aglycon intermediate, 6-deoxyerythronolide B (**6**), whereas EryK hydroxylates the C-12 position of the glycosylated compound erythromycin D (**8**) (Figure 1). Purification of these enzymes and subsequent biochemical studies in which relative substrate activities were compared revealed that minor changes in substrate structure have a profound effect on the catalytic activities of both EryF and EryK (*8, 9*).

With the development of combinatorial biosynthesis, a new technology for the production of "unnatural" natural products from genetically engineered PKSs (*10, 11*), molecular biologists and chemists are presented with opportunities to generate new anti-infective compounds by manipulating macrolide gene clusters. A growing number of novel macrolide analogues have already been produced in this manner (*12*−*17*). In many cases, however, the modified compounds lack one or more of the post-PKS hydroxylations normally catalyzed by the *eryF* (C-6 oxidation) or by the *eryK* (C-12 oxidation) gene products. Considering the apparent strict substrate specificities of these enzymes, the identification of additional macrolide monooxygenases with activities toward alternative substrates would be very useful for the oxidative "tailoring" of novel polyketide antibiotics.

Here we report the cloning of the narbomycin C-12 hydroxylase gene from *Streptomyces venezuelae* and characterization of the corresponding cytochrome P450 monooxygenase enzyme. *S. venezuelae* produces several polyketide compounds, including the 14-membered macrolides narbomycin (**1**) and picromycin (**2**) as well as the 12-membered macrolides methymycin (**4**) and neomethymycin (**5**). The hydroxylase gene, designated *picK*, was located near the putative picromycin/methymycin PKS genes. The overexpression and purification of PicK from *Escherichia coli* enabled measurement of Michaelis−Menten pa-

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[1] Abbreviations: BLAST, basic local alignment search tool; DIG, dioxigenin-dUTP; ELSD, evaporative light scattering detection; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; HPLC, high-performance liquid chromatography; LB, Luria−Bertani media; LC/MS, liquid chromatography/mass spectrometry; ORF, open reading frame; PCR, polymerase chain reaction; PKS, polyketide synthase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)-aminomethane.

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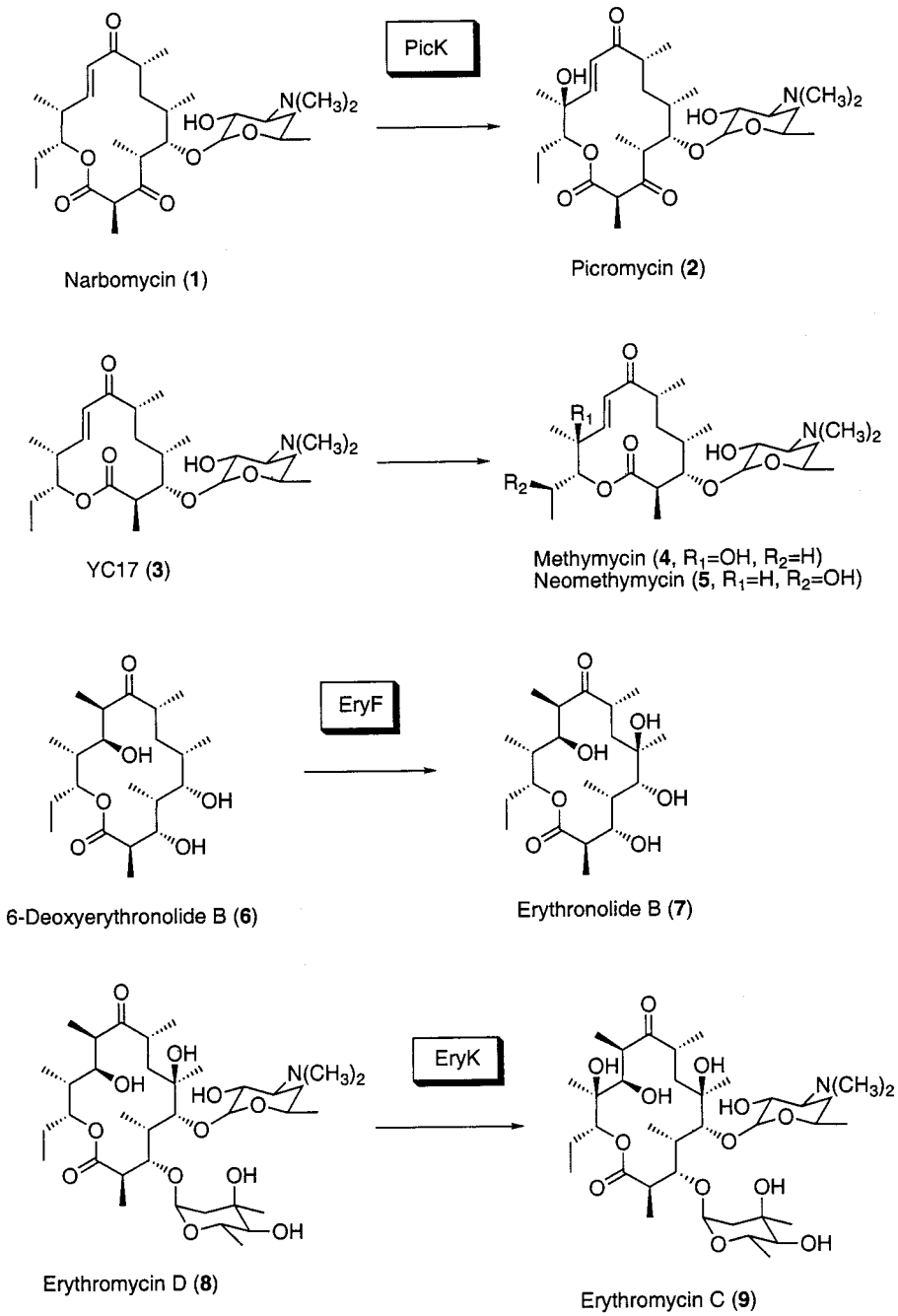

FIGURE 1: Hydroxylations catalyzed by cytochrome P450 monooxygenases in *S. venezuelae* (picromycin, methymycin, and neomethymycin) and *S. erythraea* (erythronolide B and erythromycin C). EryF catalyzes C-6 hydroxylation of an aglycon macrolide whereas both PicK and EryK catalyze C-12 hydroxylation after desosamine attachment.

rameters for the in vitro C-12 hydroxylation of narbomycin to picromycin and comparison to the oxidations performed by the two erythromycin hydroxylases. The unique substrate specificity of PicK should be useful for the engineered biosynthesis of novel ketolides (*18*), a promising class of semisynthetic macrolides with activity against antibiotic-resistant pathogens and structurally related to the natural products narbomycin and picromycin.

MATERIALS AND METHODS

Bacterial Strains, Growth, and Inducing Conditions. S. venezuelae ATCC15439, grown in tryptone soya broth (Oxoid, Hampshire, England) at 30 °C, was used as a source for genomic DNA. *E. coli* XL1-Blue (Stratagene, La Jolla, CA) was the host for DNA manipulation. For production of recombinant protein, *E. coli* BL21(DE3) harboring the appropriate expression vector was grown in LB containing carbenicillin (100 μg/mL) at 37 °C to an OD_{600} of 0.6. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and the cells were grown for an additional 3 h before harvesting.

Manipulation of DNA and Construction of Expression Plasmids. DNA manipulation, Southern hybridization, colony hybridization, and transformation of *E. coli* were performed by standard procedures (*19*, *20*). Nonradioactive labeling and detection of DNA probes were performed with PCR DIG (dioxigenin-dUTP) probe synthesis and DIG detection kits (Boehringer Mannheim, Indianapolis, IN). PCR was per-

formed with *Pfu* polymerase (Stratagene) using conditions recommended by the manufacturer.

Generation of a S. venezuelae Genomic Library and Hybridization Conditions. Genomic DNA was isolated from *S. venezuelae* using conditions described by Hopwood et al. (*19*). The DNA (100 μg) was partially digested with *Sau*3AI endonuclease to generate fragments ca. 40 kb in length. A cosmid library was prepared using the SuperCosI (Stratagene) vector as directed by the manufacturer. The ligation mixture was packaged using a GigapackIII XL packaging extract kit (Stratagene) and propagated in *E. coli* XL1-Blue MR (Stratagene). The resulting library of ca. 3000 colonies was plated on a 10 × 150 mm agar plate and replicated to a nylon membrane. The library was screened with a 1.4 kb probe prepared from *S. venezuelae* genomic DNA using degenerate ketosynthase amplifying oligonucleotides. Colonies were alkaline-lysed, and the DNA was cross-linked to the membrane using UV irradiation. After overnight incubation with the probe at 42 °C in hybridization solution (*20*), the membrane was washed twice at 25 °C in 2× SSC buffer (*20*) with 0.1% SDS for 15 min, followed by two 15 min washes with 2× SSC buffer at 55 °C. Approximately 30 colonies gave positive hybridization signals. Two representative cosmids were selected for further analysis by Southern hybridization.

The cosmids were digested with *Bam*HI and electrophoresed on a 1% agarose gel, and the resulting fragments were transferred to a nylon membrane. A cytochrome P450 probe was generated by PCR with primers specific for amplification of the entire 1.2 kb *S. erythraea* C-12-hydroxylase gene, *eryK*. The membrane was incubated with the *eryK* probe overnight at 42 °C in hybridization solution and washed twice at 25 °C in 2× SSC buffer with 0.1% SDS for 15 min, followed by two 15 min washes with 2× SSC buffer at 50 °C. One cosmid, pKOS023−26, contained a 3.0 kb fragment which hybridized with the probe under these conditions. This fragment was excised from the gel, isolated, and subcloned into the PCR-script (Stratagene) cloning vector to yield plasmid pKOS023−28. The insert was sequenced by primer walking using standard fluorescent dye terminator protocols.

Construction of PicK E. coli Expression Plasmids. The *picK* gene was PCR-amplified using oligonucleotide primers (forward, 5′-TTGCATGCATATGCGCCGTACCCAGCAGGGAACGACC; reverse, 5′-TTGAATTCTCAACTAGTACGGCGGCCCGCCTCCCGTCC). These primers alter the *Streptomyces* GTG start codon to ATG and introduce a *Spe*I site at the C-terminal end of the gene, resulting in the substitution of a serine for the terminal glycine amino acid residue. Following subcloning of the PCR product, the 1.3 kb gene fragment was cloned into the *Nde*I/*Xho*I sites of the T7 expression vector pET22b (Novagen, Madison, WI) to generate pKOS023−61. Subsequently, a short linker fragment encoding six histidine residues and a stop codon was introduced into the *Spe*I site. Thus, the final expression vector (pKOS023−68) encodes a six-His affinity-tagged version of PicK.

Preparation of Cell Extracts and Purification of PicK/6-His. All purification steps were performed at 4 °C. *E. coli* cell pellets were suspended in 32 μL of cold binding buffer (20 mM Tris/HCl, pH 8.0, 5 mM imidazole, 500 mM NaCl) per milliliter of culture and lysed by sonication. For analysis of *E. coli* cell-free extracts, the cellular debris was removed by low-speed centrifugation, and the supernatant was used directly in assays. For purification of PicK/6-His, the supernatant was loaded (0.5 mL/min) onto a 5 mL HiTrap Chelating column (Pharmacia, Piscataway, NJ) equilibrated with binding buffer. The column was washed with 25 mL of binding buffer, and the protein was eluted with a 35 mL linear gradient (5−500 mM imidazole in binding buffer). Column effluent was monitored at 280 and 416 nm. Fractions corresponding to the 416 nm absorbance peak were pooled and dialyzed against storage buffer (45 mM Tris/HCl, pH 7.5, 0.1 mM EDTA, 0.2 mM DTT, 10% glycerol). The purified 46 kDa protein was analyzed by SDS−PAGE using Coomassie blue staining. Enzyme concentration and yield were determined by Bradford assay (*21*) using bovine serum albumin as a standard.

Kinetic Assays with Narbomycin. Narbomycin (**1**) was purified from a culture of *Streptomyces narbonensis* ATCC 19790. Reactions for kinetic assays (100 μL) consisted of 50 mM Tris/HCl (pH 7.5), 100 μM spinach ferredoxin, 0.025 unit of spinach ferredoxin:$NADP^+$ oxidoreductase, 0.8 u of glucose-6-phosphate dehydrogenase, 1.4 mM $NADP^+$, 7.6 mM glucose 6-phosphate, 20−500 μM narbomycin substrate, and 50−500 nM PicK. The reaction proceeded at 30 °C, and samples were withdrawn for analysis at 5, 10, 15, and 90 min. Reactions were stopped by heating to 100 °C for 1 min, and denatured protein was removed by centrifugation. Depletion of narbomycin and formation of picromycin (**2**) were determined by high-performance liquid chromatography (HPLC, Beckman C-18 0.46 × 15 cm column) coupled to atmospheric pressure chemical ionization (APCI) mass spectroscopic detection (Perkin-Elmer/Sciex API 100) and evaporative light scattering detection (Alltech 500 ELSD).

RESULTS

Cloning and Sequencing of the picK Hydroxylase Gene. Since genes required for biosynthesis of polyketides are clustered in *Streptomyces* genomes, we pursued the isolation of the narbomycin C-12 hydroxylase by first identifying PKS genes in a cosmid library prepared from *S. venezuelae*. Cosmids hybridizing to a PKS ketosynthase probe were divided into two classes based on restriction digest patterns. Reasoning that the narbomycin hydroxylase gene would encode a macrolide cytochrome P450 enzyme, a representative cosmid from each group was further screened by Southern hybridization using the 1.2 kb *eryK* gene encoding the erythromycin C-12 hydroxylase as a probe. One of the cosmids, pKOS023−26, contained a 3.0 kb *Bam*HI fragment which hybridized strongly to the *eryK* probe. This band was subcloned, sequenced, and found to contain the entire narbomycin hydroxylase gene.

BLAST (*22*) analysis of the sequence revealed significant homology to *eryK* and other macrolide cytochrome P450 hydroxylases. A 1.3 kb open reading frame (ORF) was designated *picK*. The putative start codon for *picK* was selected on the basis of codon composition (*23*) and is located 6 nt downstream of a putative ribosome binding site. There are no other potential start codons for at least 120 nt in either direction. The translated amino acid sequence of PicK is compared to other macrolide P450 enzymes in Figure 2. As expected, PicK contains the conserved signature sequences associated with cytochrome P450 enzymes, specifically an

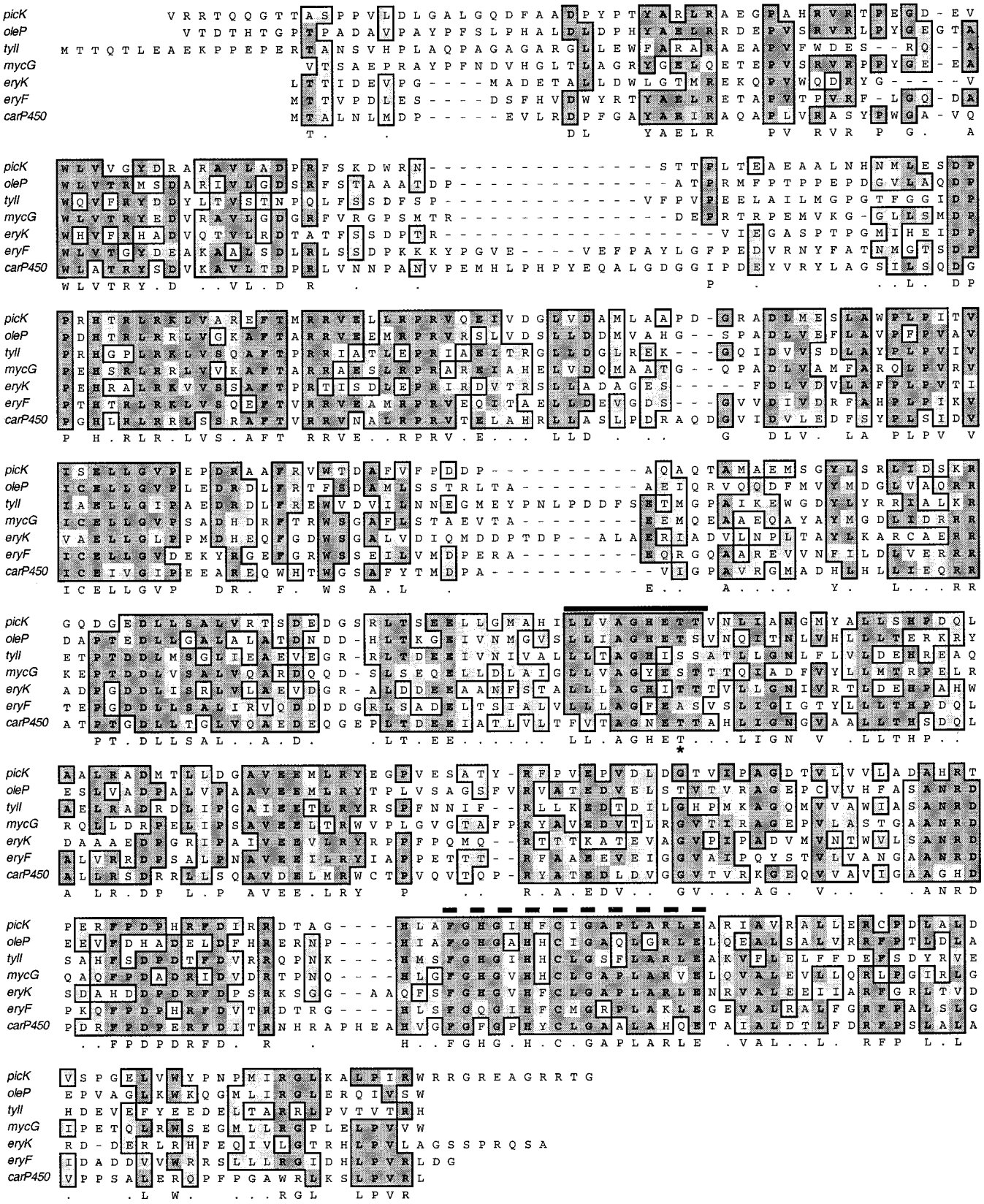

FIGURE 2: Alignment of PicK to other macrolide bacterial P450 monooxygenases. A clustalW alignment was performed using the software package MacVector (Oxford Molecular Group) with default settings. The solid line indicates the O_2 binding region with the conserved threonine/serine (alanine in EryF) residues (asterisk) believed to be involved in O_2 scission. The dashed line indicates the heme binding pocket including the conserved cysteine residue. *oleP*, putative oleandomycin 8,8a-epoxidase (*6*); *tylI*, tylosin C-20 oxidase (*4*); *mycG*, mycinamicin oxidase (5); *eryK*, erythromycin C-12 hydroxylase (*2*); *eryF*, erythromycin C-6 hydroxylase (*1*, *3*); *carP450*, putative carbomycin P450 oxidase (*29*).

O_2 binding site and a distal heme binding domain containing a highly conserved cysteine residue. Although both PicK and EryK catalyze hydroxylation at the same macrolide C-12 carbon center, PicK exhibits better homology to the C-6

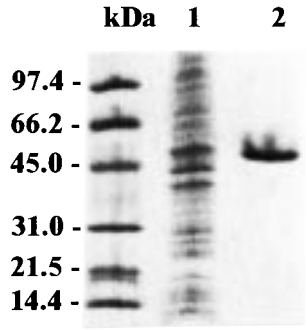

FIGURE 3: SDS−PAGE of crude *E. coli* lysate expressing PicK/6-His (lane 1) and after purification by Ni^{2+} chromatography (lane 2).

hydroxylase EryF (ca. 39% amino acid identity) rather than EryK (ca. 30% amino acid identity). The mechanism of substrate-assisted catalysis proposed for EryF is not likely to occur with PicK, however, since the conserved threonine/serine residue which is absent in the O_2 binding site of EryF is present in PicK (Figure 2) (*24*, *25*).

PicK Catalyzes the C-12 Hydroxylation of Narbomycin. The role of PicK in picromycin (**3**) biosynthesis was investigated by expressing the gene in *E. coli* and using a cell-free extract to hydroxylate the substrate narbomycin. The *picK* gene was placed under control of a T7 promoter in the expression vector pET22b (plasmid pKOS023−61). Induction of *E. coli* BL21(DE3)/pKOS023−61 with IPTG resulted in the production of a soluble 46 kDa protein consistent with the predicted molecular mass of PicK as determined by SDS−PAGE analysis (data not shown). This band was absent in a control culture of BL21(DE3) containing the vector plasmid pET22b with no insert.

The hydroxylase activity of the cell-free extract was assayed in a reaction mixture using a narbomycin substrate concentration of 200 μM. After proceeding for 90 min at 30 °C, the reaction mixture was analyzed by liquid chromatography/mass spectrometry (LC/MS). The reaction contained a compound not present in the control that displayed the same retention time, molecular weight ($M+H^+ = 526$), and mass fragmentation pattern as authentic picromycin obtained from *S. venezuelae*. The conversion of narbomycin to picromycin under these conditions was greater than 90% as estimated by ELSD.

Kinetic Measurements of PicK/6-His. To simplify purification of the PicK hydroxylase for kinetic analysis, the carboxy-terminal glycine of PicK was replaced with six histidine residues to generate a six-His affinity-tagged protein (PicK/6-His). Purification of PicK/6-His (1.4 mg from 250 mL of media broth) was achieved with a single chromatographic step on a Ni^{2+} column. The purified PicK/6-His eluant contained only a single detectable band on a Coomassie blue stained SDS−PAGE gel (Figure 3). The UV absorbance spectrum of the purified protein contained the characteristic 390−440 nm cytochrome P450 peak with a maximum at 420 nm.

Initial velocities for the hydroxylation of narbomycin were determined by directly monitoring picromycin formation via LC/MS. At a substrate concentration of 200 μM, the initial rate of hydroxylation was found to correlate linearly with enzyme concentrations in the range 50−500 nM PicK/6-His. An enzyme concentration of 250 nM was selected to obtain steady-state kinetic parameters. Initial velocities were obtained with narbomycin concentrations between 20 and 500 μM. Product formation was linear for the period monitored (0−10 min). A least-squares fit to the Michaelis−Menten equation resulted in values for $K_m = 120 \pm 30$ μM and $V_{max} = 0.34 \pm 0.04$ μM/s. Assuming 100% of the enzyme preparation to be active, this would correspond to a k_{cat} value of 1.4 ± 0.2 s^{-1} and $k_{cat}/K_m = 0.012 \pm 0.003$ μM^{-1} s^{-1}.

DISCUSSION

The clustering of secondary metabolite genes has been a tremendous aid in the isolation of genes involved in polyketide biosynthesis. For example, Stassi et al. were able to identify *eryK* by chromosomal walking from the erythromycin PKS (*2*). Our work presents an even more direct approach by using a previously identified homologous gene. Since many cytochrome P450 homologues are likely to exist in polyketide-producing organisms (*26*), the preselection of PKS containing cosmids substantially enriches for the presence of genes encoding the enzymes relevant to polyketide biosynthesis. This method could also be used for the direct isolation of other polyketide-related biosynthetic genes (e.g., deoxysugar biosynthetic or attachment genes) without extensively sequencing a gene cluster.

We cloned the narbomycin C-12 hydroxylase gene from *S. venezuelae* with the expectation that it will lead to the engineered biosynthesis of ketolide intermediates such as compound **11** (Figure 4). Ketolides (**10**), characterized by a 3-ketone functionality, are a new class of semisynthetic macrolides which have shown excellent in vitro antibacterial activity against macrolide-resistant pathogens (*18*, *27*, *28*). These compounds are currently prepared from erythromycin by a complex multistep chemical synthesis. A number of routes involving genetically engineered PKS gene clusters can potentially be used to biosynthetically produce novel ketolide intermediates. A necessary component for ketolide

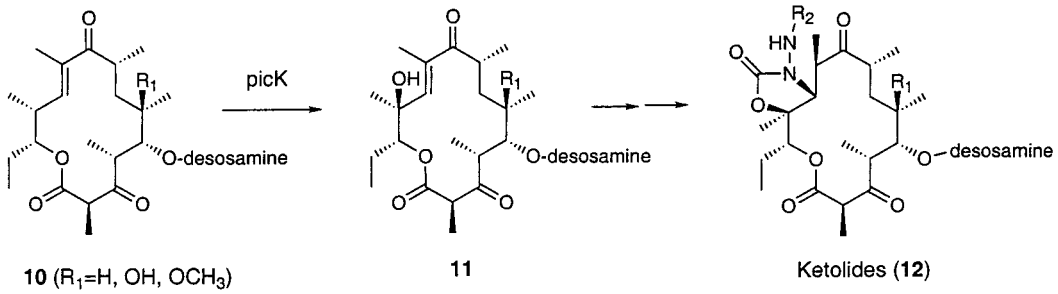

FIGURE 4: Structures of ketolides and desired biosynthetic intermediates. Attachment of carbazate substituents to ketolides requires a hydroxyl at C-12 which may be enzymatically added by PicK.

intermediates is the presence of a hydroxyl at the C-12 position for attachment of the C-11,C-12 carbazate constituents. The erythromycin C-12 hydroxylase (EryK) is not a good candidate for the hydroxylation of 3-ketolides as it has been shown to have a very stringent substrate specificity with regard to the sugar substituent at C-3 (*8*) and is inactive toward narbomycin (James Kealey, unpublished results). PicK/6-His is an excellent choice for biosynthetic preparation of ketolide intermediates, as its natural substrate contains a C-3 ketone. Efforts to prepare and confirm conversion of such substrates for the enzymatic hydroxylation by PicK/6-His are currently in progress.

PicK/6-His represents the third known macrolide P450 monooxygenase to be heterologously expressed and studied (*8*, *9*). The k_{cat} value measured for PicK/6-His is similar to the reported values of EryF ($k_{cat} = 1.7\ s^{-1}$) (*9*) and EryK ($k_{cat} = 1.8-6.3\ s^{-1}$) (*8*), while the K_m value of PicK is higher than those reported for EryF ($K_D = 2.0\ \mu M$) (*9*) and EryK ($K_m = 8-44\ \mu M$) (*8*). This represents an approximate 10−70-fold increase for the specificity constants (k_{cat}/K_m) of EryF and EryK versus PicK, almost all of which can be attributed to the lower binding affinity of PicK. While it is unknown if the six-His affinity tag addition to the C-terminus affects the measured K_m and V_{max} values of PicK, the ease of purification relative to the native enzyme greatly facilitates its use for the in vitro hydroxylation of nonnatural substrates which may require high concentrations of enzyme.

Recently, Cane and Graziani used a partially purified extract from *S. venezuelae* to demonstrate that the hydroxylation of the 12-membered lactone YC-17 (**3**) to both methymycin (**4**) and the 12-hydroxyl isomer neomethymycin (**5**) is catalyzed by a cytochrome P450 monooxygenase (*7*). Preliminary studies using YC-17 as a substrate for the enzyme reported here have demonstrated that PicK is also responsible for both of these hydroxylations in *S. venezuelae* (D. Cane, personal communication). Thus, PicK appears to possess an inherent specificity for both 12- and 14-membered macrolides which could allow the oxidative tailoring of novel macrolides with different lactone ring sizes. In addition, the apparent dual regiospecificity with regard to 12-membered lactones might be extended to perform hydroxylations at alternative positions on larger ring systems as well. The ability to further diversify natural products generated by combinatorial biosynthesis with the array of possible PKS tailoring oxidations should facilitate the search for new or improved therapeutic macrolides.

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