

Streptomyces coelicolor A3(2) CYP102 Protein, a Novel Fatty Acid Hydroxylase Encoded as a Heme Domain without an N-Terminal Redox Partner[∇]

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Received 12 December 2009/Accepted 15 January 2010

The gene from *Streptomyces coelicolor* A3(2) encoding CYP102B1, a recently discovered CYP102 subfamily which exists solely as a single P450 heme domain, has been cloned, expressed in *Escherichia coli*, purified, characterized, and compared to its fusion protein family members. Purified reconstitution metabolism experiments with spinach ferredoxin, ferredoxin reductase, and NADPH revealed differences in the regio- and stereoselective metabolism of arachidonic acid compared to that of CYP102A1, exclusively producing 11,12-epoxyeicosa-5,8,14-trienoic acid in addition to the shared metabolites 18-hydroxy arachidonic acid and 14,15-epoxyeicosa-5,8,11-trienoic acid. Consequently, in order to elucidate the physiological function of CYP102B1, transposon mutagenesis was used to generate an *S. coelicolor* A3(2) strain lacking CYP102B1 activity and the phenotype was assessed.

Streptomycetes produce a vast array of antibiotics applied in human and veterinary medicine and agriculture, as well as antiparasitic agents, herbicides, and pharmacologically active metabolites (e.g., immunosuppressants). Streptomycetes also catalyze numerous transformations of xenobiotics of industrial and environmental importance (1). The most significant of these biocatalytic reactions include aromatic and aliphatic hydroxylations, O and N dealkylations, N oxidation, and C-C coupling and fission catalyzed by heme-containing cytochrome P450 (CYP) enzyme systems (25). *Streptomyces coelicolor* A3(2) is the most-studied member of the genus in molecular genetic and biochemical investigations (1). Eighteen cytochrome P450, six ferredoxin, and four ferredoxin reductase genes were shown to be distributed across the linear chromosome, with nine P450 genes arranged in polycistronic organization with other genes (1, 14).

Arguably, the most biochemically and structurally characterized CYP to date is CYP102A1 (P450 BM3) from *Bacillus megaterium*. CYP102A1 was first isolated as a fatty acid monooxygenase in the laboratory of Armand Fulco more than 20 years ago (16). It was established as a catalytically self-sufficient monooxygenase consisting of a CYP heme domain fused at the carboxy terminus to a flavin mononucleotide (FMN)/flavin adenine dinucleotide (FAD)-containing flavin reductase domain in a single polypeptide chain. It has been proposed that such architecture affords the CYP domain optimal catalytic activity in the turnover of substrates. Indeed, of all the CYPs

characterized functionally, CYP102A1 exhibits the highest P450 turnover frequency, with rates of $>15,000 \text{ min}^{-1}$ obtained in exogenous arachidonate hydroxylation (17, 18). Furthermore, CYP102A1-like P450 reductase fusion proteins have also been found in certain fungi, including *Fusarium oxysporum* (13) and *Phanerochaete chrysosporium* (3).

CYP102B1 was the first member of a new CYP102 subfamily discovered outside *Bacillus* spp. (1) and is intriguing since it exists and functions as a single CYP protein domain. Here we report the cloning, expression, and characterization of CYP102B1 and demonstrate that the enzyme has activity in metabolizing arachidonic acid but with very different product profiles and with enzymatic rates orders of magnitude lower than those of CYP102A1. To address the question of the contribution of CYP102B1 to *S. coelicolor* A3(2) physiology, a CYP102B1 transposon mutant was generated and isolated and the phenotype of the subsequent mutant strains was analyzed.

MATERIALS AND METHODS

General methods. Reduced carbon monoxide (CO) difference spectra for quantification of cytochrome P450 content were measured and calculated according to the method described by Omura and Sato (19). Protein quantification was performed by using the bicinchoninic acid assay. Unless otherwise stated, all chemicals were supplied by Sigma Chemical Company (Poole, Dorset, United Kingdom). UV-visible absorption spectra of purified CYP102B1 were recorded using a Shimadzu UV-2401 scanning spectrophotometer as described previously (24, 26).

Cloning, gene synthesis, expression, and purification of CYP102A1 and CYP102B1. The gene for CYP102B1 was commercially synthesized using codon optimization for efficient protein expression in *Escherichia coli* and incorporating eight histidine residues at the carboxy terminus to facilitate protein purification by nickel-nitrilotriacetic acid (Ni-NTA) chromatography (DNA2.0 Inc., Menlo Park, CA 94025) and inserted into the expression vector pET17b, producing the final construct CYP102B1syn-His₈pET17b. The expression construct was transformed into *E. coli* strain BL21(DE3)pLysD, in which expression of the T7 RNA polymerase gene is under the control of the *lac* promoter. To facilitate the

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[∇] Published ahead of print on 22 January 2010.

production of correctly folded P450, CYP102B1 was coexpressed in the presence of the *E. coli* molecular chaperones GroES and GroEL as described previously (20). Three liters of *E. coli* heterologously expressing CYP102B1 was pelleted by centrifugation at $1,500 \times g$, and CYP102B1 was purified using an Ni^{2+} -NTA affinity column (Qiagen), using methods similar to those applied to the purification of other *S. coelicolor* P450s (47, 49). CYP102A1 was expressed and purified as described previously (6).

Arachidonic acid metabolism by CYP102A1 and CYP102B1. Assays of CYP102A1 and CYP102B1 arachidonic acid metabolism were performed at 30°C for 20 min. Each reaction mixture contained either 0.01 μ M CYP102A1 or 1 μ M CYP102B1, 0.01 U/100 μ l spinach ferredoxin reductase and 20 μ M spinach ferredoxin, and 70 μ M (70,000 cpm/nmol) [^{14}C]arachidonic acid. Reactions were carried out in the following reaction buffer: 0.15 N KCl, 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.4), 2 mg/ml isocitrate, and 0.1 U/ml isocitrate dehydrogenase. NADPH was added to a final concentration of 1 mM to start the reaction. Briefly, the products of the catalytic turnover of each fatty acid were determined by reverse-phase, high-performance liquid chromatography (RP-HPLC) and comparison to authentic standards (8).

Transposon mutagenesis of CYP102B1. To assess the role of *CYP102B1* in the contribution of *S. coelicolor* A(3)2 physiology and potential roles in endogenous secondary and lipid metabolism, *CYP102B1* was mutated in the *S. coelicolor* chromosome. To do so, a Tn5062 insertion (SCF43.1.A08 [http://strepdb.streptomyces.org.uk]) in *CYP102B1* (*SCO0801*) carried on cosmid SCF43 was obtained from a library of mutated cosmids (2). The transposon-tagged cosmid was initially introduced into *E. coli* strain ET12567 containing pUZ8002 (4, 21) prior to its intergeneric conjugal transfer into *S. coelicolor* (12). Mutants that had undergone allelic replacement were selected through their resistance to apramycin, conferred by Tn5062, and sensitivity to kanamycin as a consequence of the loss of the nonreplicating cosmid. The nature of the mutation was subsequently confirmed by Southern hybridization using a Tn5062-specific probe.

Growth medium for wild-type and CYP102B1 mutant strains of *S. coelicolor* A3(2) and method for lipid analysis. Phenotypic analysis of the *CYP102B1* mutant and comparison to the wild type was undertaken on different solid and liquid media as described previously (12), as was antibiotic determination. Lipid-free minimal liquid medium was used for growth of both the wild type and strains of *S. coelicolor* A3(2), as described previously (12). The medium consisted of 0.2% (wt/vol) $(NH_4)_2SO_4$, 0.5% (wt/vol) Difco Casamino Acids, 0.06% (wt/vol) $MgSO_4 \cdot 7H_2O$, 5% (wt/vol) polyethylene glycol (PEG) 6000, Minor elements solution (consisting of 0.1% [wt/vol] [each] of $ZnSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$ and $CaCl_2$ anhydrous), 1% (wt/vol) glucose, and 0.02% (vol/vol) $NaH_2PO_4 \cdot K_2HPO_4$ buffer (0.1 M, pH 6.8). Following growth at 25°C and 150 rpm for 7 days, cells were harvested by centrifugation, and lipids were extracted using the Folch method as described previously (5). Lipids were analyzed by liquid chromatography/mass spectrometry (LC/MS) using a Finnigan MAT TSO-7000 triple quadrupole mass spectrometer (Finnigan Corp., San Jose, CA) equipped with a standard electrospray ionization source outfitted with a 100- μ m-inside-diameter deactivated fused capillary. The mass spectrometer was operated in full scan mode. The data were collected from m/z 300 to m/z 900 with a 1-s scan time.

RESULTS

Cloning, expression, and purification of CYP102B1. Heterologous expression of CYP102B1syn-His₈ in *E. coli* produced extremely low levels of protein, preventing its biochemical analysis. Hence, we established an efficient system for expression with the molecular chaperones GroES and GroEL, which have been shown to enhance the production of active and correctly folded human P450s (11). Consequently, expression of active CYP102B1syn-His₈ was greatly enhanced, with levels of CYP reaching >250 nmol P450/liter culture after 44 h of culture at 15°C. Subsequent fractionations indicated that the overexpressed CYP102B1syn-His₈ was soluble and capable of generating a spectral maximum at 451 nm in reduced CO difference analysis. CYP102B1syn-His₈ was purified to near-homogeneity by a strategy similar to that described for other *S. coelicolor* P450s (24, 26), with subtle alterations. The specific content of CYP102B1syn-His₈ was approximately 12.5 nmol/mg protein. The oxidized absolute spectrum of the purified

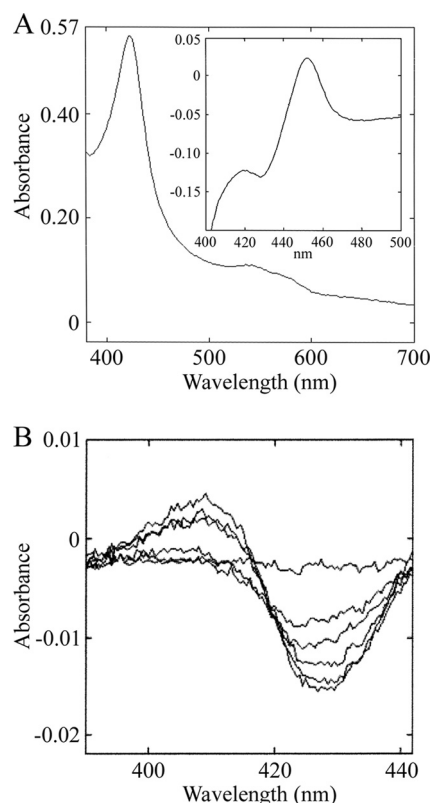


FIG. 1. Expression of recombinant CYP102B1 and spectral changes induced by binding of arachidonic acid. (A) UV-visible spectra of purified CYP102B1. Absorption spectra in resting (ferric) and carbon monoxide (CO)-bound (inset) forms are shown. All spectra were recorded at ambient temperature in 50 mM potassium phosphate buffer, pH 7.4. (B) CYP102B1 arachidonate-induced difference spectra. Various concentrations of arachidonic acid (10 μ M, 20 μ M, 30 μ M, 40 μ M, and 50 μ M dissolved in CH_3OH) were added to a solution of CYP102B1 (0.5 μ M) in 50 mM potassium phosphate buffer (pH 7.4) containing 20% (vol/vol) glycerol.

CYP102B1syn-His₈ showed a Soret band at 417 nm and α - and β -bands at 567 and 534 nm, while reduction with sodium dithionite resulted in a Soret peak at 413 nm. The Soret maximum obtained from the reduced CO difference spectrum was located at 451 nm (Fig. 1A).

Spectral characterization of fatty acid binding. Binding of fatty acids to CYP102A1 induces a shift in equilibrium of the heme iron low-spin state toward the high-spin form, leading to changes in the Soret region (18). To determine whether fatty acids bound CYP102B1, spectral binding titrations were performed with saturated (palmitic and stearic acids) and unsaturated (oleic and arachidonic acids) forms. Only arachidonic acid showed substrate-induced difference spectra (Fig. 1B). Plots of absorption changes versus the arachidonate concentration were hyperbolic, and the apparent dissociation constant, the K_d value, was determined. A clear observation is that CYP102B1 binds arachidonic acid moderately, with a K_d value calculated to be 33.3 μ M, whereas CYP102A1 binds arachidonate more tightly, with a K_d value of 3.6 μ M (18). Furthermore, palmitate, stearate, and oleate show negligible binding to CYP102B1, in contrast to CYP102A1.

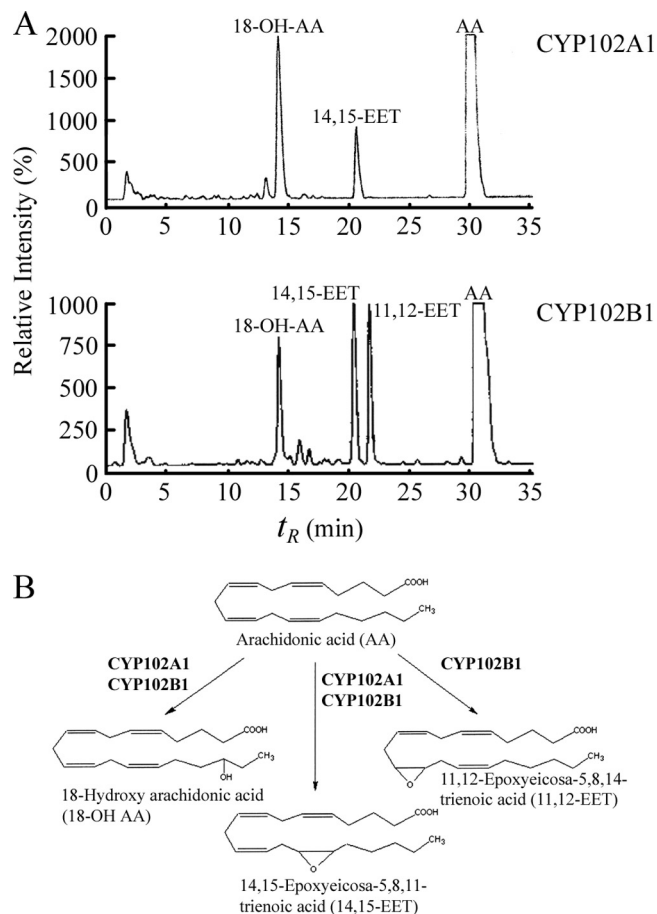


FIG. 2. Arachidonic acid hydroxylation by CYP102A1 and CYP102B1 in heterologous electron transfer systems. (A) The positions of arachidonic acid and major products are shown in each case as indicated. The concentrations of the individual protein components used were 1 μ M CYP102A1 and 1 μ M CYP102B1, 1.0 μ M spinach ferredoxin reductase, and 1.6 μ M spinach ferredoxin. Experiments were performed in triplicate, and in all control experiments (without CYP or without reductase), no product formation was observed (data not shown). (B) Metabolic pathways for the catalysis of arachidonic acid by CYP102A1 and CYP102B1.

Arachidonic acid metabolism by CYP102A1 and CYP102B1.

The turnover of arachidonic acid by CYP102A1 and CYP102B1 was determined by RP-HPLC using [14 C]arachidonic acid as a substrate, and the identities of the products were established by comparison of retention times with authentic standards and by gas chromatography-mass spectrometry (GCMS). As expected, in our experiments CYP102A1 catalyzed the metabolism of arachidonic acid with efficient hydroxylation rates of $>15,000 \text{ min}^{-1}$ (18), with two major products being observed: 18-hydroxy arachidonic acid (18-OH AA) (Fig. 2A) (retention time, 14.2 min) and 14,15-epoxyeicosa-5,8,11-trienoic acid (14,15-EET) (Fig. 2A) (retention time, 20.5 min). Analysis of catalytic activity for CYP102B1 revealed marked differences in the ratio of products produced and the production of a new product compared to results for CYP102A1, 11,12-epoxyeicosa-5,8,14-trienoic acid (11,12-EET) (Fig. 2A) (retention time, 22 min). 18-OH AA and 14,15-EET eluted at identical retention times in all experiments. CYP102B1 produced similar amounts of 11,12-EET and 14,15-EET, as well as 18-OH AA, with a catalytic rate of $20 \pm 3 \text{ min}^{-1}$,

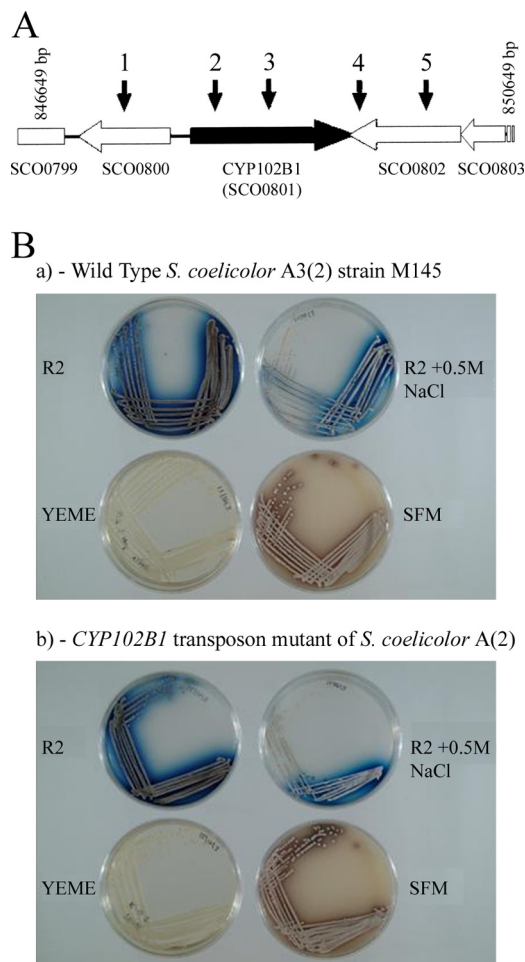
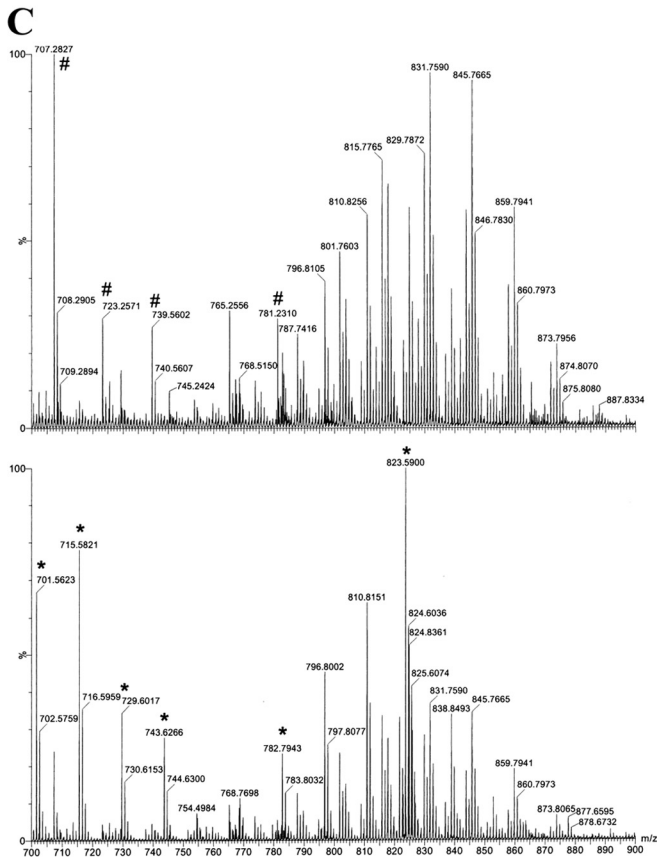
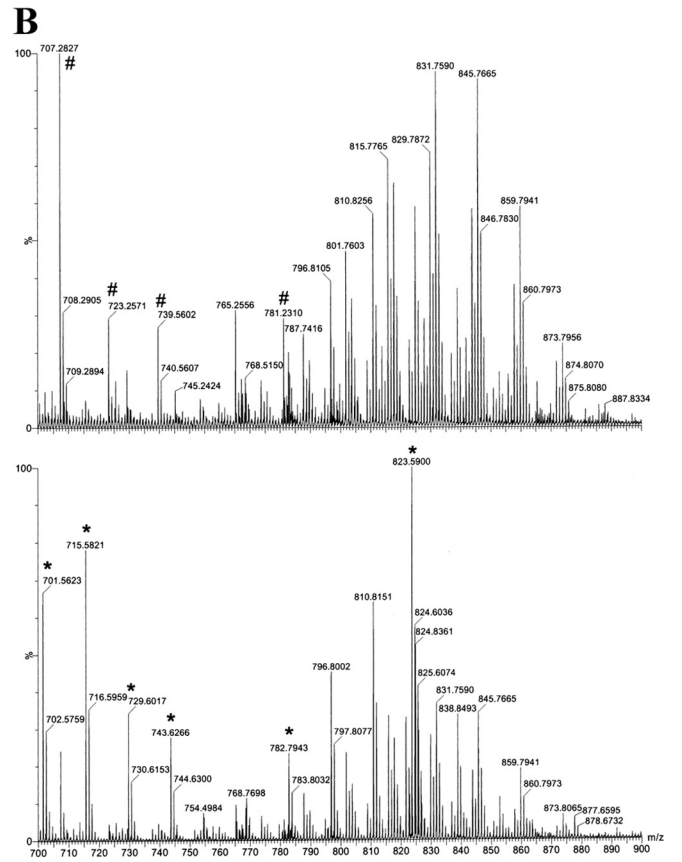
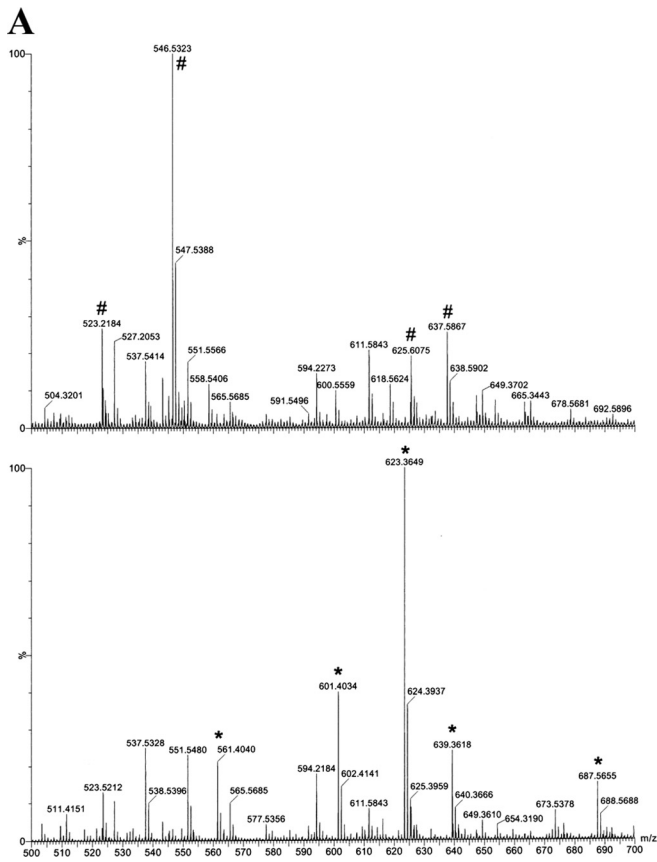


FIG. 3. (A) Structure of the CYP102B1 gene and position of the transposon insertions. Arrows above the CYP102B1 gene region indicate the location of representative Tn5062 insertions. (B) CYP102B1 is not essential for growth in *S. coelicolor* A(2). a, wild type M145 *S. coelicolor* A(2); b, CYP102B1 transposon mutant of *S. coelicolor* A(2). Cultures were incubated for 7 days at 30°C. R2 agar medium containing 10% (wt/vol) sucrose, 1% (wt/vol) glucose, 0.5% (wt/vol) yeast extract, and 0.01% (wt/vol) Casamino Acids; YEME agar medium containing 34% (wt/vol) sucrose, 1% (wt/vol) glucose, 0.3% (wt/vol) yeast extract, 0.3% (wt/vol) malt extract, and 0.5% (wt/vol) Bacto-peptone; and SFM agar medium containing 2% (wt/vol) soya flour and 2% (wt/vol) mannitol were used for growth of both the wild-type and CYP102B1 strains of *S. coelicolor* A3(2), as described previously (41).

whereas CYP102A1 produced approximately 60% 18-OH AA and 40% 14,15-EET, with a catalytic rate of $3,200 \pm 400 \text{ min}^{-1}$. The production of 11,12-EET by CYP102B1 is unique to this CYP102 family member, since no CYP102A has been shown to produce this molecule from arachidonic acid (Fig. 2B).

CYP102B1 is not required for normal cell growth and secondary metabolite production. A CYP102B1 transposon mutant (insertion SCF43.1.A08; <http://strepdb.streptomyces.org.uk>) was generated, and lipids from the mutant strain were extracted, analyzed, and compared to the lipids from the parent strain. The location of the Tn5062 insertion within the CYP102B1 gene was 463 bp from the 5' end (following amino acid G¹⁵⁴, 285 residues prior to the conserved cysteine located at amino acid



position 440) and 1,120 bp from the 3' end of the gene, i.e., 5' to the part of the gene encoding the heme binding domain (Fig. 3A). The heme-binding domain is located at ⁴³³FGTGARACIG⁴⁴². Since this domain is essential for any CYP catalytic activity, no functional CYP102B1 was produced in the mutant.

No phenotypic differences were observed between the mutant and the M145 parental strain in comparing both antibiotic production and cell development when they were grown under different solid medium conditions (Fig. 3B). Similarly, *B. subtilis* CYP102A2 and CYP102A3 were previously shown to be nonessential (9). Given the overwhelming evidence in the literature documenting fatty acid metabolism by members of the CYP102 family, particularly CYP102A1, and our own data establishing CYP102B1 as a fatty acid hydroxylase, we were led to investigate the lipid profiles of both the parent and mutant strains.

Using wild-type and CYP102B1 mutant whole-cell lipid extracts from 1 liter of stationary-phase cultures, a variety of previously identified and new conjugated lipids were observed. These included phospholipids, glycolipids, lipoamino acids, glycerides, and fatty acids. Careful examination of the lipid profiles from both the parent and CYP102B1 mutant lipid extracts revealed differences in the lipids produced between strains (Fig. 4A to C). Although there were quantitative differences in the lipids isolated between experiments, the qualitative differences observed in the lipid profiles were always consistent. Due to the complexity of the many new classes of previously unidentified *S. coelicolor* fatty acids and lipids which are observed, including a range of hydroxyl fatty acids, deciphering the basis of these changes is not possible at this time. Given that there are clear alterations in the lipid profile following disruption of the CYP102B1 gene, a role for the gene product in lipid biochemical pathways is strengthened. However, further work in the long term will be required to decipher these alterations.

DISCUSSION

To date, 27 members of the CYP102 family have been uncovered and named. First, it is important to note that all CYP102 genes have been found exclusively in soil-dwelling bacteria, suggesting that they confer a metabolic advantage to these particular organisms in this environment. Second, 14

belong to the CYP102A subfamily. CYP102A6 was identified in the nitrogen-fixing bacterium *Bradyrhizobium japonicum*, which is the only Gram-negative bacterium to date to possess a CYP102 gene. The remaining 13 CYP102s belong in different subfamilies (B to J). Further, the majority of CYP102s are fusion proteins with a P450 heme domain and FMN/FAD-containing reductase domain, with only *S. coelicolor* CYP102B1, *Streptomyces avermitilis* CYP102B2, *Rhodococcus* sp. RHA1 CYP102B3, and *Streptomyces scabies* CYP102B4 existing as a P450 domain alone in a single polypeptide chain (15). Only three CYP102 P450s have been biochemically analyzed to date, CYP102A1, A2, and A3 (10). Even comparing these three members in the same subfamily, differences in catalytic activity were reported, with CYP102A2 and A3 from *Bacillus subtilis* showing high selectivity for long-chain unsaturated and branched fatty acids compared with results for *B. megaterium* CYP102A1 (10).

In *S. coelicolor*, CYP102B1 is not associated genetically with other genes unlinked in the chromosome. This is also true for CYP102B2 in *S. avermitilis*. To assess the contribution of CYP102B1 to the life cycle of *S. coelicolor*, we isolated a strain carrying a transposon mutation of this gene. Earlier work using deletion of CYP102A2 and CYP102A3 individually or combined has shown that they are not essential to the life cycle of *B. subtilis* (9). Disruption of CYP102B1 in *S. coelicolor* had no effect on the ability of the organism to grow and differentiate compared to the wild type, ruling out roles for CYP102B1 in cell development. We then explored the mutation of CYP102B1 for secondary metabolite production and the production of the two pigmented antibiotics actinorhodin and undecylprodigiosin in particular. There was no difference in the generation and yield of the two antibiotics on comparing the mutant with the wild type. With no phenotypic differences observed in a comparison of the wild-type and CYP102B1 mutant *S. coelicolor* strains, one cannot exclude enzymatic redundancy of CYP102B1 in the genome of *S. coelicolor*, but given the high selectivity and substrate specificity for P450s in general and CYP102s in particular, this would be highly unlikely.

In biotechnology, there is much potential for application of CYPs demonstrable in past use, such as the 11 β -hydroxylation of Reichstein S in the biosynthesis of hydrocortisone and the formation of dicarboxylic acids from alkanes (22). Such reactions are catalyzed by fungal fermentations and P450 monooxygenases. Consequently, several laboratories have used iso-

FIG. 4. (A) Positive-ion electrospray mass spectra (m/z 500 to m/z 700) of CH₂Cl-methanol extracts of lipids from wild-type *Streptomyces coelicolor* A3(2) (top) or the CYP102B1 mutant (bottom). High-resolution mass spectra were determined by direct liquid infusion using a Waters, Inc., Synapt Q-TOF instrument operating in reflectron time-of-flight (TOF) mode at resolution 9,000 (peak width at half-height). Samples were infused at 10 μ l/min in chloroform-acetonitrile-isopropanol-water (30:35:30:5) with 5 mM ammonium acetate. Lipid species in wild-type *S. coelicolor* differing from those of the mutant are identified by the “#” symbol (top); conversely, changes in the CYP102B1 mutant from the wild-type are denoted by “*” (bottom). The lipids differing between the wild-type and mutant strains were not readily identifiable by searching the Lipid MAPS database (<http://www.lipidmaps.org/index.html>). (B) Positive-ion electrospray mass spectra (m/z 700 to m/z 900) of CH₂Cl-methanol extracts of lipids from wild-type *Streptomyces coelicolor* A3(2) (top) or the CYP102B1 mutant (bottom). Lipid species in wild-type *S. coelicolor* differing from those of the mutant are identified by the “#” symbol (top); conversely, changes in the CYP102B1 mutant from the wild type are denoted by “*” (bottom). The lipids differing between the two organisms were not readily identifiable by searching the Lipid MAPS database (<http://www.lipidmaps.org/index.html>). (C) Negative-ion electrospray mass spectra (m/z 580 to m/z 910) of CH₂Cl-methanol extracts of lipids from wild-type *Streptomyces coelicolor* A3(2) (top) or the CYP102B1 mutant (bottom). High-resolution mass spectra were determined by direct liquid infusion using a Waters, Inc., Synapt Q-TOF instrument operating in reflectron TOF mode at resolution 9,000 (peak width at half-height). Samples were infused at 10 μ l/min in chloroform-acetonitrile-isopropanol-water (30:35:30:5) with 5 mM ammonium acetate. Lipid species in wild-type *S. coelicolor* differing from those of the mutant are identified by “#” (top); conversely, changes in the CYP102B1 mutant from the wild type are denoted by “*” (bottom). The lipids differing between the two organisms were not readily identifiable by searching the Lipid MAPS database (<http://www.lipidmaps.org/index.html>).

lated P450 enzymes, and CYP102A1 in particular, to investigate novel enzymatic activities with applied use. For example, site-directed mutagenesis of CYP102A1 led to an enzyme with activity toward testosterone and the drugs dextromethorphan and amodiaquine (23), while random mutagenesis led to a CYP102A1 mutant with alkane hydroxylase activity (7). Herein, we have characterized a new member of the CYP102 family which has unique stereo- and regiospecific catalysis toward a known CYP102A1 substrate and structurally consists only of the heme domain, whereas all CYP102s known to date comprise a fusion between the heme domain and a redox partner. It will be of interest in the future to understand the key molecular features, including specific amino acid residues, underpinning these enzymatic differences and to generate a fusion protein of the CYP102B1 heme domain with a suitable reductase partner in order to exploit this enzyme fully for biotechnological purposes. Such work will be undertaken in our laboratories in the future.

ACKNOWLEDGMENTS

Support was provided by National Institutes of Health grants R01GM69970 and P30ES00267 and by a Leverhulme Trust Research Fellowship and a Royal Society Travel Award.

We appreciate the advice and experimental support of Jorge Capdevila and S. Wei.

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