

Investigating conservation of the albaflavenone biosynthetic pathway and CYP170 bifunctionality in streptomycetes

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Keywords

albaflavenone; cytochrome P450; enzyme bifunctionality; evolution; *Streptomyces*

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(Received 22 July 2011, revised 1 December 2011, accepted 6 December 2011)

doi:10.1111/j.1742-4658.2011.08447.x

Albaflavenone, a tricyclic sesquiterpene antibiotic, is biosynthesized in *Streptomyces coelicolor* A3(2) by enzymes encoded in a two-gene operon. Initially, sesquiterpene cyclase catalyzes the cyclization of farnesyl diphosphate to the terpenoid *epi*-isozizaene, which is oxidized to the final albaflavenone by cytochrome P450 (CYP)170A1. Additionally, this CYP is a bifunctional enzyme, being able to also generate farnesene isomers from farnesyl diphosphate, owing to a terpene synthase active site moonlighting on the CYP molecule. To explore the functionality of this operon in other streptomycetes, we have examined culture extracts by GC/MS and established the presence of albaflavenone in five *Streptomyces* species. Bioinformatics examination of the predicted CYP170 primary amino acid sequences revealed substitutions in the CYP terpene synthase active site. To examine whether the terpene synthase site was catalytically active in another CYP170, we characterized the least related CYP170 orthologue from *Streptomyces albus* (CYP170B1). Following expression and purification, CYP170B1 showed a normal reduced CO difference spectrum at 450 nm, in contrast to the unusual 440-nm peak observed for *S. coelicolor* A3(2) CYP170A1. CYP170B1 can catalyze the conversion of *epi*-isozizaene to albaflavenone, but was unable to catalyze the conversion of farnesyl diphosphate to farnesene. Molecular modeling with our crystal structure of CYP170A1 suggests that the absence of key amino acids for binding the essential terpene synthase cofactor Mg^{2+} may be the explanation for the loss of CYP170B1 bifunctionality.

Introduction

Streptomycetes are Gram-positive, soil-dwelling filamentous bacteria that owe their survival within a complex environment to numerous physiological mechanisms [1]. These include the ability to produce an array of catabolic enzymes that can degrade biopolymers, and the ability to biosynthesize mixtures of antimicrobial compounds, allowing them to protect

their nutrients from competitors and hence promote growth [2]. Many of these important natural antibiotics and bioactive compounds are currently used in medicine and agriculture [3]. *Streptomyces coelicolor* A3(2), the model species, has been used to study morphological differentiation and antibiotic regulation, and is known to generate at least five antibiotics:

Abbreviations

CYP, cytochrome P450; FPP, farnesyl diphosphate; TRP, tetratricopeptide repeat protein.

actinorhodin, prodigiosins, calcium-dependent antibiotic, methylenomycin, and albaflavenone [2].

The tricyclic sesquiterpene albaflavenone was first isolated from *Streptomyces albidoflavus*, and is known to exhibit antibacterial properties [4]. The pathway for the biosynthesis of albaflavenone was recently elucidated in *S. coelicolor* A3(2) [5]. Its production requires expression of a two-gene operon encoding a sesquiterpene cyclase (SCO5222) and a cytochrome P450 (CYP) (SCO5223; CYP170A1), with the *sco5223* gene sharing a four-nucleotide ATGA translational overlap with the *sco5222* gene. Biochemically, SCO5222 catalyzes the cyclization of farnesyl diphosphate (FPP) to the hydrocarbon *epi*-isozizaene, through initial ionization and isomerization of FPP generating the tertiary allylic intermediate, (3*R*)-nerolidyl diphosphate, followed by a rearrangement cascade to ultimately produce *epi*-isozizaene (Fig. 1) [6]. Subsequently, CYP170A1 carries out two sequential allylic oxidations to convert *epi*-isozizaene to an epimeric mixture of albaflavenols and ultimately to the single ketone sesquiterpene, the antibiotic albaflavenone (Fig. 1) [5]. SCO5222 contains two conserved Mg^{2+} -binding domains in its primary sequence that are essential for catalytic activity, and that are found in all sesquiterpene synthases [6]. The first is an aspartate-rich **DDRHD**(99–103) motif and the second the characteristic NSE triad, **NDLCSLPKE**(240–248), found in SCO5222. Mutagenesis of these key residues, either individually or collectively, leads to almost complete loss of enzymatic activity [6,7].

Furthermore, incubation of CYP170A1 with FPP alone resulted in the formation of the terpenes (*E*)- β -farnesene (61%), (3*E*,6*E*)- α -farnesene (26%), (3*Z*,6*E*)- α -farnesene (6.8%), nerolidol (4.9%), and farnesol (1.8%), suggesting a dual-function enzyme [8]. Subsequently, the crystal structure of CYP170A1 revealed the presence of a novel terpene synthase active site, which is moonlighting on the CYP structure [8]. This includes signature sequences for divalent cation binding (the aspartate-rich DDXD and the NSE triad) and a distinctive α -helical barrel. This barrel is unusual, because it consists of only four helices rather than the six found in all other terpene synthases. Mutagenesis established that this barrel is essential for the terpene synthase activity of CYP170A1 but not for the albaflavenone synthase activity [8]. This was the first moonlighting CYP to be discovered with two distinct catalytic active sites in a single protein molecule.

We now report the albaflavenone biosynthetic pathway to be more highly conserved than any other antibiotic pathway in *Streptomyces* species. BLAST searches, with the *S. coelicolor* A3(2) SCO5222 and CYP170A1 protein sequences as queries, revealed likely orthologous proteins present in *Streptomyces lividans*, *Streptomyces viridochromogenes*, *Streptomyces sviveus*, *Streptomyces avermitilis*, *Streptomyces ghanaensis*, *Streptomyces griseoflavus*, *Streptomyces* sp. E14, *Streptomyces* sp. SPB74, *Streptomyces* sp. SPB78, and *Streptomyces albus*. Chemical extraction and GC/MS confirmed that five species (*S. viridochromogenes*, *S. avermitilis*, *S. griseoflavus*, *S. ghanaensis*, and *S. albus*), in addition to

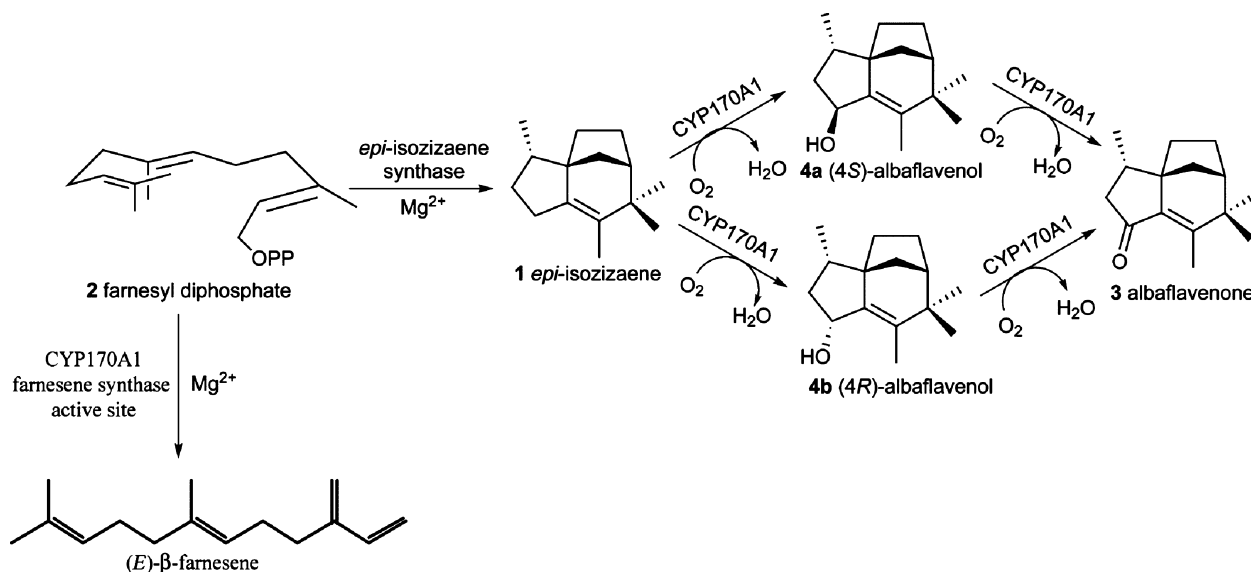


Fig. 1. The albaflavenone and farnesene biosynthetic pathway in *S. coelicolor* A3(2).

S. coelicolor A3(2), biosynthesize albaflavenone. Additionally, we investigated CYP170 bifunctionality through a combination of bioinformatics analysis, molecular modeling, and biochemical characterization of the least sequence related CYP170 ortholog (CYP170B1) from *S. albus*. Our results suggest a more important and possibly generalized role regarding the biological function of the albaflavenone biosynthetic operon. Finally, the CYP170 family can be divided into two subfamilies at present: CYP170A enzymes, which are albaflavenone monooxygenases and predicted to be bifunctional enzymes [as previously shown for *S. coelicolor* A3(2) CYP170A1] [8]; and CYP170B enzymes, which are also albaflavenone monooxygenases but are not predicted to be bifunctional enzymes (as shown herein for *S. albus* CYP170B1).

Results

Identification of CYP170 orthologs in streptomycetes by bioinformatics analysis

Takamatsu *et al.* used a bioinformatics approach to identify orthologous genes encoding *epi*-isozizaene synthase and CYP170 in nine bacterial strains, and indicated that all but one is translationally coupled to a downstream CYP gene [9]. Each putative *epi*-isozizaene synthase was identified by the high degree of protein similarity between all proteins and the retention of canonical motifs essential for *epi*-isozizaene synthase activity identified in the resolved crystal structure of the *S. coelicolor* A3(2) enzyme. Concurrently, we undertook a bioinformatics search to determine the presence of other bifunctional CYPs in the databases. Hence, a BLAST search with the protein sequence of the *S. coelicolor* A3(2) CYP, CYP170A1, as a query identified 10 CYPs with homology to CYP170A1 in the following streptomycete genomes: *S. lividans*, *S. viridochromogenes*, *S. sviveus*, *S. avermitilis*, *S. ghanaensis*, *S. griseoflavus*, *Streptomyces* sp. E14, *Streptomyces* sp. SPB74, *Streptomyces* sp. SPB78, and *S. albus*. All CYPs identified contained the signature CYP motifs used for identification: the heme-binding domain **FXGXRX**CXX and the **EXXR** motif involved in stabilization of heme during incorporation and CYP folding. Amino acid sequence alignment of seven CYP170 family members revealed a high degree of identity with CYP170A1 (*S. lividans*, 99%; *S. viridochromogenes*, 86%; *S. ghanaensis*, 81%; *S. sviveus*, 80%; *S. avermitilis*, 80%; *S. griseoflavus*, 75%; and *Streptomyces* sp. E14, 74%). Additionally, protein sequence alignment revealed conservation of the terpene synthase active site moonlighting on each of

the seven CYPs in an analogous fashion to CYP170A1. All seven CYPs contain the conserved Mg²⁺-binding motifs [**DDXX**TXXXE(250(242–250) and **DDXXD**(253–257) in CYP170A1)], which form part of a four-helical barrel responsible for terpene synthase activity, suggesting that all of these CYPs are bifunctional. The only variation found in the Mg²⁺-binding motifs of six of the seven CYPs is in the ortholog from *S. sviveus*, which contains the changes **DDXX**TXXXA(237–245) and **DAXXD**(248–252), but these are not predicted to alter Mg²⁺ binding, owing to the conservative nature of both substitutions. The least related CYP170 orthologs coupled to the *epi*-isozizaene synthase gene were found in *Streptomyces* sp. SPB74, *Streptomyces* sp. SPB78, and *S. albus*, each showing approximately 56% identity with CYP170A1. Within these three CYP170 orthologs, the Mg²⁺-binding motifs, which form the moonlighting terpene synthase site in CYP170A1, show differences from those in the other CYP170A orthologs, and these CYPs represent a new CYP170 subfamily – CYP170B. In the *S. albus* CYP170 (CYP170B1) sequence, the Mg²⁺ motifs are **DDXXDXXX**T(236–244) and **DGX**XR(247–251), and there is reduced similarity in the α -helical barrel, suggesting that CYP170B1 may not be bifunctional. Similarly, in *Streptomyces* sp. SPB74 and *Streptomyces* sp. SPB78 CYP170Bs, the Mg²⁺ motifs are **DDXXSXXX**H(240–248) in both, and **GEX**XE(251–255) in *Streptomyces* sp. SPB74 and **GEX**XE(251–255) in *Streptomyces* sp. SPB78, again suggesting a loss of bifunctionality.

Furthermore, examination of the genes surrounding the albaflavenone operon in each streptomycete reveals a high degree of conservation. Directly upstream, the *sigR* and anti-*sigR* genes encode sigma factors that coordinate expression of the disulfide stress regulon. Additional genes that are highly conserved in each species include a gene that encodes an integral membrane protein that may play a role in albaflavenone export and a gene that encodes a tetratricopeptide repeat protein (TRP); TRPs constitute a family of proteins that have been shown to form scaffolds to mediate protein–protein interactions [10]. TRP may be involved in forming a complex between *epi*-isozizaene synthase and CYP170, allowing efficient production of albaflavenone through the channeling of *epi*-isozizaene between both enzymes.

Biosynthesis of albaflavenone in streptomycetes

The conservation of the albaflavenone biosynthetic operon revealed by bioinformatics analysis led us to focus on the identification of albaflavenone in

additional streptomycetes. Takamatsu *et al.* [9] confirmed the production of *epi*-isozizaene in three *Streptomyces* species, *S. ghanaensis*, *S. lividans*, and *S. albus*, with only albaflavene being detected in *S. albus*. No detectable amounts of either *epi*-isozizaene or albaflavene were formed in *S. avermitilis*, *S. sviveus* and *S. viridochromogenes* under their experimental conditions. In our experiments, culture extracts from *S. lividans*, *S. viridochromogenes*, *S. avermitilis*, *S. ghanaensis*, *S. griseoflavus* and *S. albus* were also analyzed by GC/MS for the production of albaflavene. As shown in Fig. 2, we were able to confirm that *S. ghanaensis*, *S. avermitilis*, *S. griseoflavus*, and *S. viridochromogenes*, as well as *S. albus*, do produce albaflavene. The *m/z* 218 peak was identified as albaflavene by direct comparison of the retention time and mass spectral fragmentation pattern with those of the authentic albaflavene (Fig. 2). Hence, in contrast to Takamatsu *et al.*, we show that the albaflavene operon is directly functional in four additional streptomycetes as well as in *S. coelicolor* A3(2) and *S. albus*.

Expression and spectral characterization of *S. albus* CYP170B1

To investigate the conservation of the bifunctionality in CYP170 orthologs, the least related ortholog in relation to *S. coelicolor* A3(2) CYP170A1, from *S. albus*, CYP170B1, was expressed and purified. Similarly to the previous expression of CYP170A1, CYP170B1

produced negligible levels of correctly CYP when expressed directly in *Escherichia coli* under the control of the *lac* promoter in pET17b (Novagen, Merck KGaA, Darmstadt, Germany). Therefore, correctly folded CYP was produced by coexpressing it with the molecular chaperones GroES and GroEL, which have been shown to enhance the production of a range of active and correctly folded CYPs [11]. In this coexpression system, GroES and GroEL coding regions are under the control of the *AraB* promoter, allowing them to be induced by the addition of arabinose. In the presence of these proteins, expression of active CYP170B1 was greatly enhanced, with levels of CYP reaching > 500 nmol CYP/L culture after 72 h of culture. Cell fractionation revealed CYP170B1 to be a soluble protein, being located in the cytosolic fraction following ultracentrifugation at 100 000 *g*. No CYP was detected in the isolated *E. coli* membranes. After two consecutive Ni²⁺-affinity chromatography purification steps a homogeneous band was observed on SDS/PAGE at approximately 55 kDa, as compared with the predicted molecular mass of the protein of 50.19 kDa (Fig. 3A). Purified CYP170B1 had a Soret (γ) maximum at 417.5 nm, and virtually equal intensities for the distinguishable α (571.5 nm) and β (535 nm) bands, indicating the CYP to be in the oxidized enzymatically active form, with the majority of the heme iron in the low-spin state (Fig. 3B). Additionally, purified CYP170B1 has a typical CYP-reduced CO spectrum, with the spectral maximum at

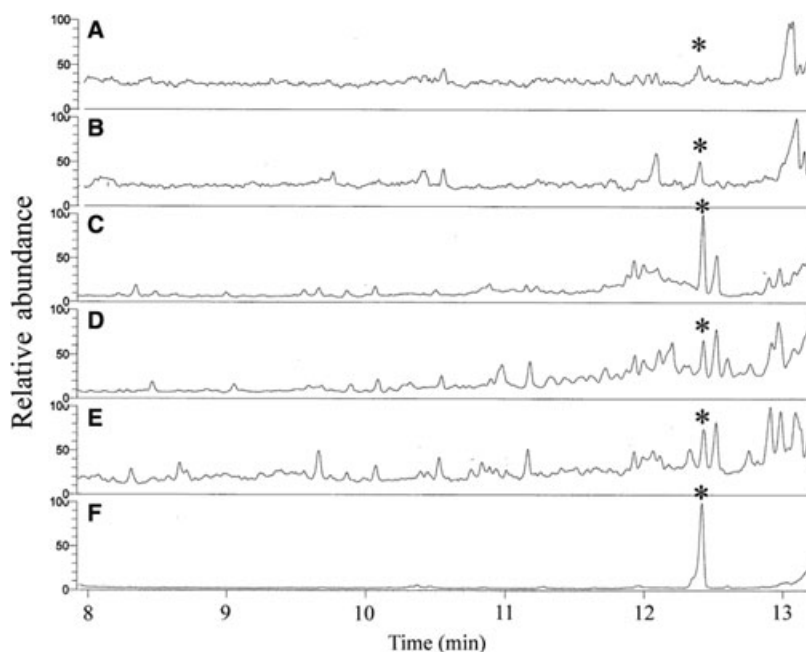


Fig. 2. GC/MS analysis of streptomycete extracts identifying the presence of albaflavene. GC traces of compounds produced by: (A) *S. ghanaensis*, (B) *S. avermitilis*, (C) *S. griseoflavus*, (D) *S. viridochromogenes*, and (E) *S. albus*, and (F) authentic albaflavene. The albaflavene peak is indicated by an asterisk (*).

450 nm (Fig. 3B, inset). In contrast, the expressed *S. coelicolor* A3(2) CYP170A1 in *E. coli* cytosol extracts and the purified protein display a unique CO difference spectrum, with a maximum at 440 nm [5]. The molecular basis for the highly unusual Soret maximum at 440 nm in CYP170A1 is still unexplained.

Involvement of *S. albus* CYP170 in albaflavenone biosynthesis and investigation of sesquiterpene synthase bifunctionality

The turnover of *epi*-isozizaene by CYP170B1 and the identities of the products were established by GC/MS, with authentic albaflavenone as a standard. Additionally, control reactions with CYP170A1 were carried out in parallel (data not shown). CYP170B1 efficiently

catalyzed the conversion of *epi*-isozizaene (retention time, 12.3 min) to albaflavenone, *m/z* 218 (retention time, 13.8 min; Fig. 4). As the endogenous redox partners for CYP170B1 are not known, catalytic activity was supported by the *E. coli* flavodoxin and flavodoxin reductase system, which was previously shown to support CYP170A1 activity. Negative control incubations lacking CYP170 or reducing equivalents did not generate any products (data not shown). As expected, in positive control reactions with CYP170A1, *epi*-isozizaene was converted to albaflavenone via an alcohol intermediate, as previously described [5]. However, only one albaflavenol intermediate was detected under the incubation conditions used for CYP170B1-catalyzed conversion of *epi*-isozizaene to albaflavenone (retention time, 12.9 min; Fig. 4), rather than an

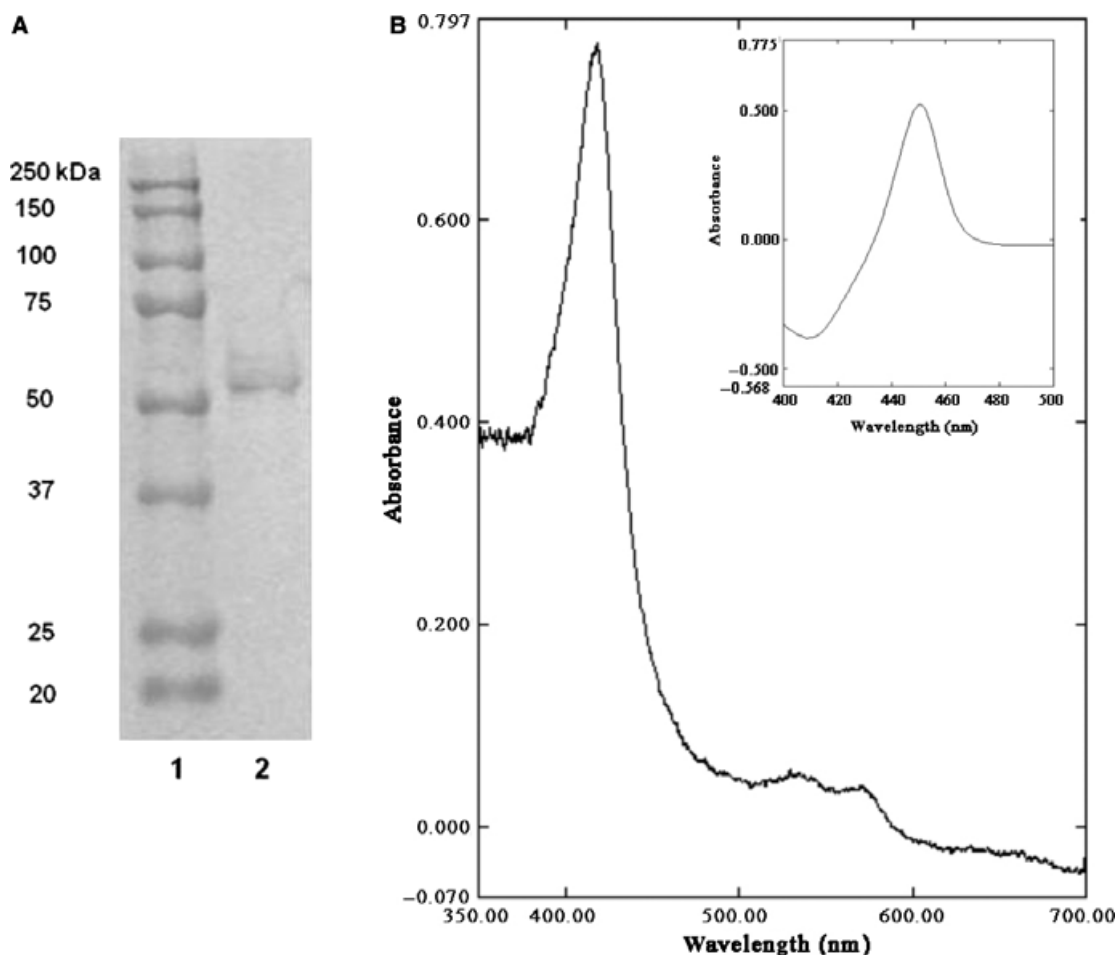


Fig. 3. SDS/PAGE and spectral analysis of purified CYP170B1 ortholog. (A) 12% PAGE. Lane 1: Precision Plus protein kaleidoscope standards marker (BioRad, Hemel Hempstead, UK). Lane 2: purified CYP170B1 after Ni^{2+} -nitrilotriacetic acid chelating chromatography. (B) The oxidized absolute spectrum of CYP170B1 (1 μM); the inset shows the reduced CO difference spectrum, which shows a classical reduced CO difference spectrum at 450 nm in comparison to that for *S. coelicolor* A3(2) CYP170A1, where the Soret peak is at 440 nm [27]. Spectra were recorded as described in Experimental procedures.

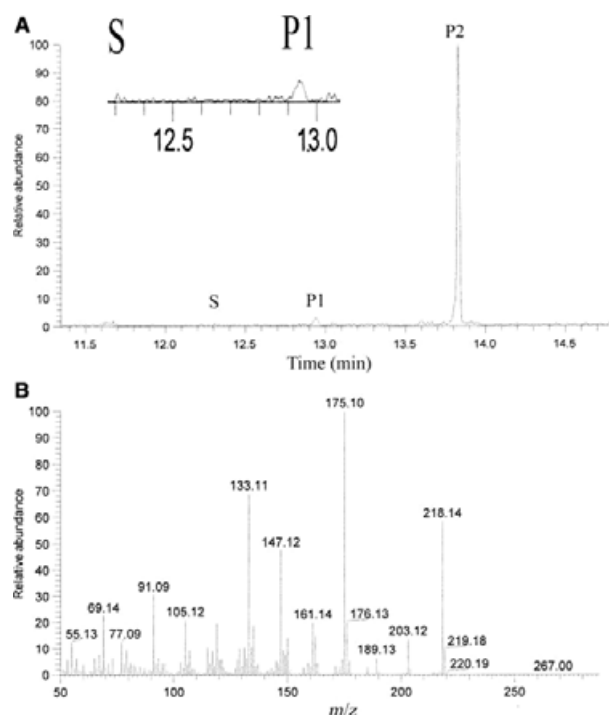


Fig. 4. *In vitro* catalytic activity of CYP170B1 supported by flavodoxin and flavodoxin reductase. Incubations were carried out as described previously [28]. (A) Product profile obtained with CYP170B1 (1 nmol), flavodoxin (10 nmol), flavodoxin reductase (2 nmol), and *epi*-isozizaene (40 nmol). *Epi*-isozizaene is present (S), as well as one minor albaflavenol product (P1) and the major albaflavenone product (P2) (see also inset). (B) The mass spectrum of product P2 showing m/z 218. This spectrum was identical to the spectrum for purified albaflaveone.

epimeric mixture of albaflavenols as seen for CYP170A1. The crystal structure of CYP170A1 suggested that *epi*-isozizaene could bind in two different orientations within the active site, and this was proposed to be the basis for the two alcohol intermediates being seen.

Additionally, it has been shown that purified CYP170A1 displays terpene synthase activity and can catalyze the conversion of FPP to the sesquiterpene hydrocarbons (*E*)- β -farnesene, (3*E*,6*E*)- α -farnesene, (3*Z*,6*E*)- α -farnesene, nerolidol, and farnesol [8]. To test whether CYP170B1 was a bifunctional CYP, we undertook similar experiments with this protein, using FPP as a substrate. GC/MS analysis of the reaction mixture of CYP170B1 incubated with FPP in the presence of Mg^{2+} did not reveal the generation of any M^+ m/z 204 terpenes, suggesting that CYP170B1 does not have an intrinsic terpene synthase activity, as is found for CYP170A1. For CYP170A1, it has been shown that the optimal pH for farnesene synthase activity is between pH 5.5 and pH 6.5. Hence, we

examined farnesene synthase activity at pH 5.5 and pH 7.4. However, no detectable formation of terpene products was seen at each pH value. Indeed, CYP170B1 was sensitive to changes in pH. At low pH, CYP170B1 became denatured and was converted to the P420 form. Furthermore, in our hands, this protein was only stable at pH 7.4, which has been described as the optimal pH for activity of CYP170A1 in albaflavenone biosynthesis. Together, these results further strengthen the conclusion that CYP170B1 is not a bifunctional CYP in an analogous fashion to CYP170A1.

Discussion

Streptomyces produce a vast array of antibiotics that are applied in human and veterinary medicine and agriculture, as well as for herbicides, pharmacologically active metabolites (e.g. immunosuppressants), and several enzymes important in the food and other industries [2,3]. Consequently, there is much interest in the sequencing and annotation of individual streptomyces genomes as a means of uncovering biosynthetic pathways, assigning pathways to known secondary metabolites, and uncovering cryptic pathways for novel molecules. To date, four streptomyces genomes have been sequenced, annotated, and published in the literature: *S. coelicolor* A3(2) [2], *S. avermitilis* [12], *Streptomyces griseus* IFO 13350 [13], and *S. griseus* strain XylebKG-1 [14]. Additionally, the draft genomes of a further five streptomyces genomes have recently been published [15–19], and further unpublished sequences have been deposited in various databases. Hence, there is now a wealth of information to be mined regarding the multitude of secondary metabolite gene clusters found in streptomyces as well as how streptomyces are evolving.

Bioinformatics analysis and comparison of all the completed streptomyces genomes reveal common features. Generally, each streptomyces has a unique secondary metabolic armory specific to that particular species. Although the classes of streptomyces natural products are generalized – for example, genomes encode pathways for the biosynthesis of terpenes, polyketides, siderophores, etc., and a specific species may have one or multiple numbers of these pathways – each streptomyces biosynthesizes structurally distinct chemical entities. However, analysis of the distribution of terpene biosynthetic pathways in streptomyces suggests conservation of sesquiterpene synthase genes in multiple streptomyces. For example, geosmin biosynthesis is widely conserved in many streptomyces [20]. It is believed that sesquiterpenes play signaling

which is necessary for catalysis. The short β turn and helix start in CYP170A1 presents four negatively charged residues that allow coordination of Mg^{2+} close to the diphosphate group of FPP (Fig. 5A). The equivalent loop region is extended in CYP170B1, and consequently adopts a more relaxed conformation. Importantly, the region in CYP170B1 also lacks two of the four negatively charged residues present in the Mg^{2+} -binding site of CYP170A1 (Fig. 5B). Specifically, CYP170A1 residues Arg116, Glu263 and Asp253 are conserved in CYP170B1 (Arg110, Glu257 and Asp247, respectively), but CYP170A1 residues Asp254 and Asp257 are replaced by Gly248 and Arg251 in CYP170B1. These changes are thought to have a

Structural modeling and subsequent comparison of our determined crystal structures of CYP170A1 [8] and CYP170B1 described in this work provides a strong rationale for the observed functional differences with respect to the inactive terpene synthase active site in CYP170B1. In CYP170A1, the binding sites for FPP and Mg^{2+} are oriented favorably for coordination of the Mg^{2+} with the diphosphate group of FPP,

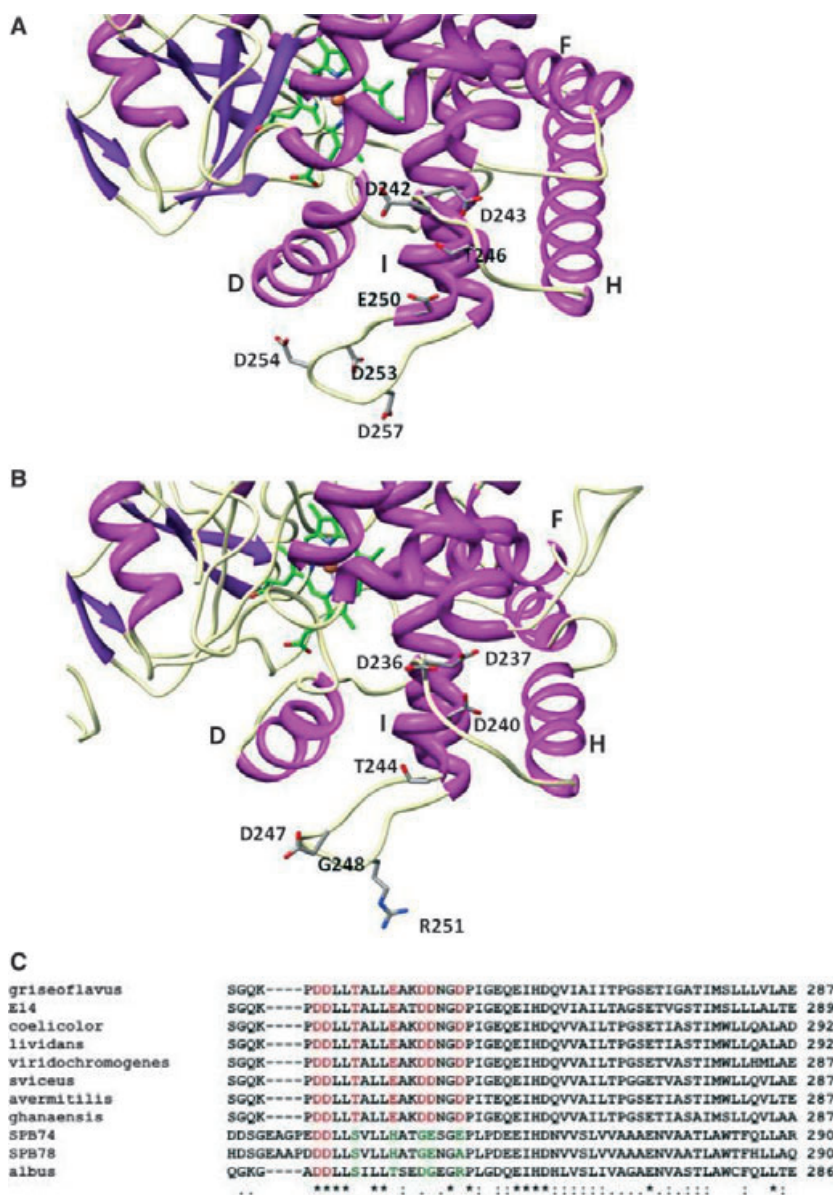


Fig. 5. Structural modeling of CYP170B1 with the crystal structure of CYP170A1. (A) The *S. coelicolor* A3(2) CYP170A1 binding sites for FPP and Mg^{2+} , with the heme group shown in green and prominent helices labeled. The Mg^{2+} -binding site, formed by negatively charged residues (Asp242, Asp243, Glu250, Asp253, Asp254, and Asp257) along with Thr246, is located at the end of the long I helix and on the adjacent β turn loop, allowing coordination of the Mg^{2+} with the diphosphate group of FPP in catalysis. (B) The equivalent region in *S. albus* CYP170B1. The β turn loop is extended, adopts a more relaxed conformation, and lacks two of the six negatively charged residues present in the Mg^{2+} -binding site of CYP170A1. In addition, a positive charge (R251) is introduced into the R region. It is probable that such substitutions lead to loss of Mg^{2+} and thus catalytic activity towards FPP. (C) Sequence alignment of the sesquiterpene synthase motifs responsible for CYP170A1 bifunctionality shows that this motif is present in the eight CYP170A homologs but not in the three CYP170B forms.

severe detrimental effect on Mg^{2+} binding, and consequently farnesene synthase activity. In particular, the replacement of the negatively charged aspartate with the positively charged arginine is predicted to greatly affect cation (Mg^{2+}) cofactor binding. We conclude that such substitutions are responsible for the loss of bifunctionality in CYP170B1. This result is consistent with a CYP170A1 mutant containing the amino acid substitutions AAXXA in the Mg^{2+} binding site resulting in loss of farnesene synthase activity [8]. Sequence alignment and examination of the Mg^{2+} -binding region of all 11 CYP170 orthologs indicate that, as well as CYP170B1, the CYP170B orthologs from *Streptomyces* sp. SPB74 (CYP170B2) and *Streptomyces* sp. SPB78 (CYP170B3) are unlikely to be bifunctional, given they also show differences in key residues responsible for Mg^{2+} binding (Fig. 5C).

To conclude, the functionality of the albaflavenone biosynthetic gene cluster in a number of streptomycetes has been verified, and bioinformatics analysis has confirmed the presence of this pathway in a number of others, as previously described [9]. Bioinformatics analysis of the CYP170 orthologs from the two-gene operon suggests that the CYP170A subfamily is predicted to encompass bifunctional enzymes in an analogous fashion to *S. coelicolor* A3(2) CYP170A1. In particular, sequence analysis reveals the retention of the Mg^{2+} -binding sites and α -helical barrel, which forms the terpene synthase active site in CYP170A1. However, the least related ortholog, CYP170B1 from *S. albus*, was shown to be able to convert *epi*-isozizaene to albaflavenone *in vitro*, but was not able to produce farnesene from FPP. Hence, this is not a bifunctional enzyme like CYP170A1, and this can be explained by the absence of key amino acids involved in binding Mg^{2+} in CYP170A1, which are essential for farnesene synthase activity. This is also probably the case for the CYP170B orthologs from *Streptomyces* sp. SPB74 and *Streptomyces* sp. SPB78. Key future experimental goals will be to establish farnesene biosynthesis *in vivo*, its physiological role, and why CYP170A bifunctionality is conserved in comparison with the CYP170B subfamily.

Experimental procedures

Bacterial strains, bioinformatics, and protein modeling

S. coelicolor A3(2) M145 and the $\Delta\text{CYP170A1}$ knockout strain were utilized as previously described [22]. *S. lividans* and *S. avermilitis* were obtained from the ATCC culture collection (<http://www.lgcstandards-atcc.org/>). *S. albus*

ATCC 2396 was kindly supplied by the John Innes Centre. *S. ghanaensis* ATCC 14672, *S. griseoflavus* Tu4000 and *S. viridochromogenes* DSM 40736 were obtained from the Broad Institute, MA, USA (<http://www.broadinstitute.org/>). A BLAST homology search of all known streptomycete genomes was performed with the protein sequences of *S. coelicolor* A3(2) *epi*-isozizaene synthase (SCO5222; UniProt [Q9K499](#)) and CYP170A1 (SCO5223; UniProt [Q82IV2](#)). CYP170B1 structural modeling was carried out with a homology modeling pipeline built with the BISKIT structural bioinformatics platform [23]. T-COFFEE was used for alignment of the test sequence [24], and homology models were generated over 10 iterations of the MODELLER program [25].

Isolation and identification of albaflavenone synthesized by streptomycetes

All *Streptomyces* strains were cultured at 30 °C in yeast extract/malt extract medium for 7 days at 200 r.p.m. [7]. The mycelia from 500-mL fermentation cultures were harvested by centrifugation for 30 min at 3500 g, and the cell pellet was extracted three times with 30 mL of pentane/dichloromethane (4 : 1). After separation of phases, the upper organic layer was collected, dried over Na_2SO_4 for 20 min on ice, concentrated under a stream of N_2 , and subjected to GC/MS as described previously [5]. Authentic albaflavenone was purified from *S. albidoflavus* with methods described previously [4]. Briefly, cells were cultured at 30 °C in yeast extract/malt extract medium for 5 days at 200 r.p.m. Harvested cells from 2 L of culture were acidified by addition of concentrated HCl to pH 6.6, and extracted three times into an equal volume of ethyl acetate. Albaflavenone was purified from the combined extract solvent by HPLC on a 5- μm silica column (10 $\times$ 150 mm; Zorbax Rx-SIL, Agilent Technologies, Santa Clara, CA, USA). Elution was undertaken with a linear solvent gradient from 65% of ethyl acetate (solvent A) to 100% of hexane (solvent B) over 25 min at a flow rate of 1.5 mL $\cdot$ min $^{-1}$, monitored with UV detection at 254 nm. Each fragment was examined by GC/MS, and the purified albaflavenone was pooled and stored at -20 °C.

Cloning, heterologous expression and purification of *S. albus* CYP170

Genomic DNA from *S. albus* was isolated according to previously described procedures [26]. Primers SA170F (5'-GCGCATATGAGCGCCGAATCCACCA-3') and SA-170R (5'-GCGAAGCTTTCATGGTGATGGTGCGGC TCTCGACCCGGAA-3') were designed to amplify the *S. albus* CYP170B1 ORF. The primers incorporate unique *Nde*I (underlined) and *Hind*III (double underlined) cloning sites and a C-terminal polyhistidine tag (bold) to allow purification of the expressed protein by affinity chromato-

graphy. PCR conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 30 cycles of 95 °C for 20 s, 57 °C for 30 s and 68 °C for 4 min, followed by a final extension step at 68 °C for 7 min. The integrity of the *S. albus* CYP170B1 was confirmed by DNA sequencing. *S. albus* CYP170B1 was expressed and purified with the use of similar conditions as previously described [5]. To improve the production of correctly folded CYP protein, CYP170B1 was coexpressed with the molecular chaperones GroES and GroEL [11]. Briefly, cotransformed cells were cultured overnight in LB broth containing 100 µg·mL⁻¹ ampicillin and 50 µg·mL⁻¹ kanamycin. After inoculation (1 : 100) in 3 L of Terrific Broth containing 100 µg·mL⁻¹ ampicillin and 50 µg·mL⁻¹ kanamycin, growth was carried out at 37 °C and 200 r.p.m. for 6 h. Following the addition of 1 mM aminolevulinic acid for heme synthesis, CYP expression was induced by the addition of 1 mM isopropyl thio-β-D-galactoside, and chaperone expression was induced by addition of arabinose to a final concentration of 4 mg·mL⁻¹. Cell growth was continued for an additional 72 h at 27 °C and 170 r.p.m. Subsequently, the cells were harvested by centrifugation at 1500 g for 20 min, resuspended in lysis buffer (250 mM sucrose, 50 mM Tris/HCl, pH 7.4, 0.5 mM EDTA), and incubated with 1 mg·mL⁻¹ lysozyme for 30 min on ice prior to freezing at -80 °C. Cells were broken by freeze-thawing and sonication as previously described, and the cytosolic fractions were separated from cell debris and the membrane fraction by ultracentrifugation at 100 000 g for 1 h. The soluble CYP170B1 was purified by metal (Ni²⁺) affinity chromatography (Qiagen, Crawley, UK), with established methods [5].

CYP activity assays *in vitro*

Farnesene synthase activity and albaflavenone synthase reactions were carried out as previously described [28], and activities were investigated at two different pH values (5.5 and 7.4), with suitably adjusted buffers. For albaflavenone production, CYP170B1 (1 nmol) was reconstituted with *E. coli* flavodoxin (10 nmol) and *E. coli* flavodoxin reductase (2 nmol), as previously described [8]. The enzymatic reactions were carried out for 5 min in 10-mL test tubes in a shaking water bath at 30 °C. Reactions were stopped and extracted following addition of a pentane/dichloromethane mixture (4 : 1). Extracts were concentrated under a stream of N₂ and analyzed by GC/MS. In each experiment, CYP stability was assessed, including reduced CO difference spectrum analysis.

General methods

Reduced CO difference spectra for quantification of CYP content were measured and calculated according to the method described by Omura and Sato [27]. Protein purity was assessed by SDS/PAGE [28] and specific CYP content.

Unless otherwise stated, all chemicals were supplied by Sigma Chemical Company (Poole, UK). UV-visible absorption spectra of purified CYPs were recorded with a Hitachi U-3310 scanning spectrophotometer.

Acknowledgements

We thank P. Dyson and R. Del Sol for helpful advice and discussion. This work was supported by National Institutes of Health Grant R01 GM69970 (to M. R. Waterman), a Biotechnology and Biological Sciences Research Council studentship (S. C. Moody), and a Wellcome Trust Sabbatical Award (D. C. Lamb).

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