Sterol 14α-demethylase activity in *Streptomyces coelicolor* A3(2) is associated with an unusual member of the CYP51 gene family

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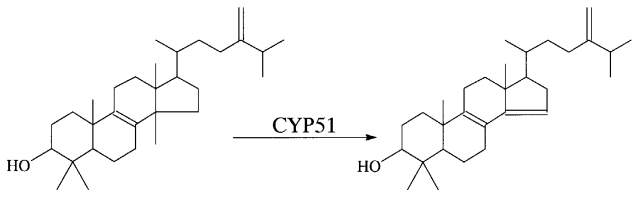
The annotation of the genome sequence of *Streptomyces coelicolor* A3(2) revealed a cytochrome P450 (CYP) resembling various sterol 14α-demethylases (CYP51). The putative CYP open reading frame (SC7E4.20) was cloned with a tetrahistidine tag appended to the C-terminus and expressed in *Escherichia coli*. Protein purified to electrophoretic homogeneity was observed to bind the 14-methylated sterols lanosterol and 24-methylene-24,25-dihydrolanosterol (24-MDL). Reconstitution experiments with *E. coli* reductase partners confirmed activity in 14α-demethylation for 24-MDL, but not lanosterol. An *S. coelicolor* A3(2) mutant containing a transposon insertion in the *CYP51* gene, which will abolish synthesis of the functional haemoprotein, was isolated as a viable strain, the first time a CYP51 has been identified as non-essential. The role of this CYP in bacteria is intriguing. No sterol product was detected in non-saponifiable cell extracts of the parent *S. coelicolor* A3(2) strain or of the mutant. *S. coelicolor* A3(2) CYP51 contains very few of the conserved CYP51 residues and, even though it can catalyse 14α-demethylation, it probably has another function in *Streptomyces*. We propose that it is a member of a new CYP51 subfamily.

Key words: cytochrome P450, evolution, sterol, *Streptomyces*, transposon.

INTRODUCTION

Since Konrad Bloch [1] put forward a biosynthetic pathway for the conversion of lanosterol into cholesterol, removal of the sterol 14α-methyl group (Scheme 1) has been the subject of much scrutiny and experimentation. This reaction is performed by a specific cytochrome P450 (CYP) enzyme, sterol 14α-demethylase (also known as CYP51 and $P450_{14DM}$), which catalyses the oxidative removal of the 14α-methyl group of lanosterol and 24-methylene-24,25-dihydrolanosterol (24-MDL) in yeast and fungi [2], obtusifoliol in plants [3] and 24,25-dihydrolanosterol in mammals [4]. The product is a $\Delta^{14,15}$-desaturated intermediate of the biosynthetic pathways of ergosterol (fungi), phytosterol (plants) and cholesterol (animals). During the catalytic cycle, a substrate undergoes three successive mono-oxygenation reactions, resulting in the formation of 14-hydroxymethyl, 14-carboxaldehyde and 14-formyl derivatives, followed by elimination of formic acid with the introduction of a 14,15 double bond [5–9].



Scheme 1 CYP-catalysed sterol 14α-demethylation step in sterol biosynthesis

CYP51 catalyses the removal of the sterol C-14 methyl group as formic acid through three sequential mono-oxygenation reactions, with the alcohol and aldehyde intermediates being produced.

The origin and evolution of the sterol biosynthetic pathway continues to be enigmatic, but is exploited in anti-fungal and anti-protozoal therapies through use of selective inhibitors of key steps or using agents which bind to membrane sterols (reviewed in [10]). Indeed, inhibition of the sterol 14α-demethylation step by the azole anti-fungal compounds is the basis of a multibillion pound industry in medicine and agriculture [11,12]. The sterol pathway itself has been proposed to be very ancient, perhaps having arisen during the later stages of microbial evolution, after the introduction of molecular oxygen into the atmosphere, as oxygen is incorporated and used in various steps [13]. For bacteria, the architectural requirements of the cell membrane can be satisfied by hopanoids synthesized directly from squalene by an anaerobic pathway [14], rather than by sterols, which are synthesized from squalene by an aerobic pathway [15]. Previously, after reflections on the genome sequence of *Mycobacterium tuberculosis* that suggested the presence of a bacterial CYP51, we observed that *M. smegmatis* contained sterol and other non-saponifiable products, unlike control *Escherichia coli* grown under the same conditions ([16] and C. J. Jackson, D. C. Lamb, N. J. Manning, D. E. Kelly and S. L. Kelly, unpublished work). Subsequently, a *CYP51* gene with sterol 14α-demethylase activity was shown to be present in *M. tuberculosis* [17].

The essential role of CYP51 in eukaryotes is clear from gene deletion experiments [18] and from the development of anti-microbial agents that target this step [10]. Hence, more detailed information on the role of bacterial CYP51 is of interest for a better understanding of the evolution of the CYP superfamily and may be of value in its consideration as a target for anti-

Abbreviations used: BSTFA, bis(trimethylsilyl)trifluoroacetamide; CYP, cytochrome P450; CYP51, sterol 14α-demethylase; NTA, nitrilotriacetic acid; ORF, open reading frame; 24-MDL, 24-methylene-24,25-dihydrolanosterol; SCCYP51, *Streptomyces coelicolor* sterol 14α-demethylase.

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mycobacterial therapy [19]. *Streptomyces coelicolor* A3(2) is another actinomycete which, on the basis of analysis of 16S rRNA gene data, appeared along with mycobacteria in the last 500 millions years [20], but is considered a model for actinomycete biology.

The annotation of the *S. coelicolor* A3(2) genome sequence predicts 18 putative CYPs (http://www.sanger.ac.uk/Projects/S-coelicolor/), one of which can be predicted to be a CYP51. In the present study, we have characterized the product of this open reading frame (ORF) (SC7E4.20). We find that it can catalyse sterol 14α-demethylase activity, yet it is not essential for growth and survival of this prokaryote. We propose, on the basis of sequence comparison, that it is a member of a new CYP51 subfamily.

EXPERIMENTAL

Cloning and heterologous expression of *S. coelicolor* sterol 14α-demethylase (SCCYP51)

Genomic DNA from *S. coelicolor* A3(2) was isolated as described previously [21]. The upstream primer, 5′-CGCCATATGACCG-TCGAGTCCGTCAAC-3′, incorporated a *Nde*I restriction site (underlined) and the initiator codon; the downstream primer, 5′-CGCAAGCTTCA**GTGATGGTGATG**CCGCGCCACGGGC-CTGAC-3′, included a *Hin*dIII restriction site (underlined). Additionally, the stop codon was removed from the gene and four histidine codons (shown in bold), followed by a new stop codon, were inserted at the 3′-end of the coding sequence. PCR conditions were 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 70 s, followed by a final extension step at 72 °C for 10 min. A final concentration of 8 % (v/v) DMSO in the PCR mixture was essential for successful gene amplification. A band of the expected size for SCCYP51 (1377 bp) was eluted from an agarose gel using the Qiagen II kit (Qiagen, Crawley, West Sussex, U.K.). The purified PCR product was digested with *Nde*I and *Hin*dIII and the DNA cloned directly into the *E. coli* expression vector pET17b (Novagen, Beeston, Nottingham, U.K.), giving SCCYP51/pET17b. The expression construct was verified by complete sequence analysis and was transformed into competent BDL(e3)pLysS cells (Novagen). Single ampicillin-resistant colonies were grown overnight at 37 °C in 5 ml of Luria–Bertani broth containing 100 μg/ml ampicillin. Such starter cultures were used to inoculate 500 ml of modified terrific broth medium supplemented with 100 μg/ml ampicillin in 2 litre flasks. Following growth for 5 h at 37 °C with shaking (250 rev./min), CYP expression was induced through addition of isopropyl β-D-thiogalactoside (1 mM final concentration). Additionally, δ-aminolaevulinic acid was added to a final concentration of 2 mM for optimal CYP synthesis. Expression was performed at 25 °C with shaking at 190 rev./min for 24 h.

Isolation and purification of SCCYP51

E. coli expressing SCCYP51 (5 litres) were pelleted by centrifugation at 1500 ***g*** and resuspended in 100 ml potassium phosphate buffer (pH 7.4). Cells were broken following two passages through a C5 homogenizer (Avestin, GlenCreston, Stanmore, Middx., U.K.) using an operating pressure of 15000 lb/in^2 (1 lb/in^2 = 6.9 kPa). The lysed cells were centrifuged at 10000 ***g*** for 20 min at 4 °C to remove unbroken cells and cell debris. The cytosolic fraction was separated from the membrane fraction by ultracentrifugation at 100000 ***g*** for 45 min at 4 °C. CYP was isolated using an Ni^{2+}-nitrilotriacetic acid (NTA) affinity column (Qiagen) equilibrated with buffer A [50 mM potassium phosphate (pH 7.4) and 20 % (v/v) glycerol]. The column was washed with buffer A containing 50 mM glycine and 0.5 M NaCl, and the CYP was eluted with buffer A containing 40 mM L-histidine.

Reconstituted CYP51 catalytic activity

Each reaction mixture contained 1 nmol/ml purified SCCYP51, 8 nmol of *E. coli* flavodoxin, 4 nmol of *E. coli* flavodoxin reductase (purified as described previously [22]) and 23 nmol of lanosterol or 24-MDL. The reaction volume was adjusted to 950 μl with 100 mM potassium phosphate buffer (pH 7.2). NADPH was added to a final concentration of 1 mM to start the reaction. All reactions were incubated at 37 °C for 20 min with gentle agitation. Reactions were stopped by the addition of 3 ml of methanol, and sterols were extracted by incubation with 90 % (w/v) KOH in ethanol for 1 h at 80 °C in a preheated water bath. Following silylation for 1 h at 60 °C with 50 μl of bis(trimethylsilyl)trifluoroacetamide (BSTFA) in 50 μl of toluene, sterol substrates and metabolites were clearly separated and identified by GC–MS (VG 12-250; VG Biotech, Manchester, U.K.). The activity (nmol of demethylated product formed/min per nmol of CYP) was calculated using the amount of substrate added and the conversion ratio (calculated from the areas of the two peaks representing methylated and demethylated sterol).

Isolation of a SCCYP51 transposon insertion mutant

A mutant of SCCYP51 was isolated from a library of 1×10^5 independent mutants generated by *in vivo* transposition in *S. coelicolor* A3(2). The transposon used was Tn*4560* [23] containing a viomycin marker (K. Fowler and T. Kieser, unpublished work). Isolation and identification of the mutant strain was confirmed by PCR and DNA sequencing.

Comparison of the non-saponifiable lipid extracts of wild-type and CYP51 mutant strains of *S. coelicolor* A3(2)

Sterol-free minimal liquid medium was used for growth of both wild-type and *cyp51*$^-$ strains of *S. coelicolor* A3(2), as described previously [21]. The medium consisted of 0.2 % (w/v) $(NH_4)_2SO_4$, 0.5 % (w/v) Difco Casaminoacids, 0.06 % (w/v) $MgSO_4.7H_2O$, 5 % (w/v) polyethylene glycol 6000, Minor elements solution [consisting of 0.1 % (w/v) of each of $ZnSO_4.7H_2O$, $FeSO_4.7H_2O$, $MnCl_2.4H_2O$ and anhydrous $CaCl_2$], 1 % (w/v) glucose, 0.02 % (v/v) NaH_2PO_4/K_2HPO_4 buffer (0.1 M, pH 6.8). Following growth at 25 °C and 150 rev./min for 7 days, cells were harvested by centrifugation and non-saponifiable lipids were extracted. Cell pellets were washed and resuspended in 5 ml of methanol, 2 ml of 0.5 % (w/v) pyrogallol in methanol and 3 ml of 60 % (w/v) potassium hydroxide in water, and incubated at 90 °C for 2 h in a preheated water bath. Following silylation for 1 h at 60 °C with 50 μl of BSTFA in 50 μl of toluene, compounds were separated and analysed by GC–MS.

General methods

Reduced CO-difference spectra for quantification of CYP content were measured and calculated as described by Omura and Sato [24]. Substrate-induced spectral changes were examined as described by Jefcoate [25]. Protein quantification was performed by using the bicinchoninic acid assay (Sigma, Poole, Dorset, U.K.). Sterols were kindly supplied by Dr B. C. Baldwin and Dr A. J. Corran (Zeneca Agrochemicals, Jeallotts Hill, Berks., U.K.). Unless otherwise stated, all chemicals were supplied by Sigma.

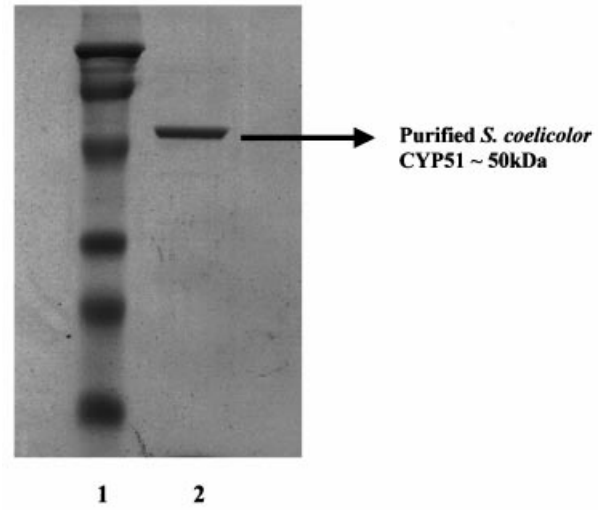

Figure 1 Protein purification analysis showing the isolation of SCCYP51, as monitored by Coomassie Brilliant Blue staining

SCCYP51 was purified to electrophoretic homogeneity through His-tag purification using Ni^{2+}-NTA affinity chromatography. Lane 1, molecular-mass markers (97.4, 66, 45, 29, 23 and 14 kDa); lane 2, CYP eluate obtained following two passes over Ni^{2+}-NTA.

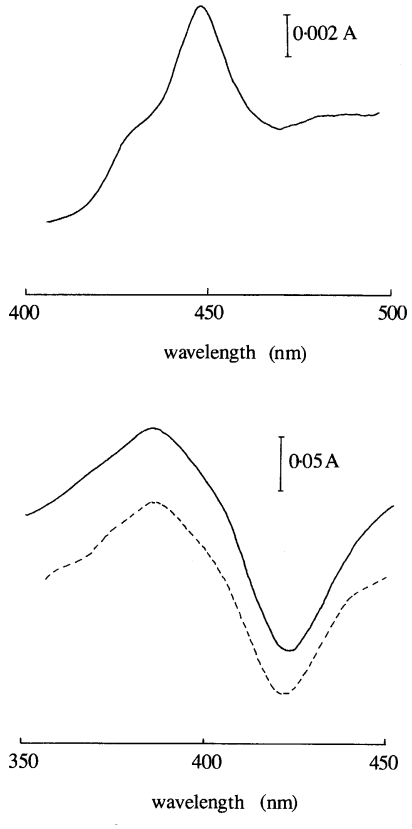

Figure 2 CO-difference spectrum and substrate-binding spectra of purified SCCYP51

Upper panel: the spectrum was recorded with sodium dithionite-reduced SCCYP51 (0.2 nmol) in each cuvette after bubbling the sample cuvette with CO for 1 min. Lower panel: each cuvette contained 500 μl of purified SCCYP51 (1 nmol). Lanosterol (—) and 24-MDL (- - -) were added to the measuring cuvette at a final concentration of 5 μM. The sterol-binding spectra were recorded at a scan speed of 1 nm/s. All spectra were corrected for the absorbance contribution of the substrate. The bars represent absorbance (A) change.

RESULTS

Cloning and heterologous expression of SCCYP51 in *E. coli*

SCCYP51 was heterologously expressed in *E. coli* (< 10 nmol/l) and had a typical CYP reduced CO-difference spectrum, with a spectral maximum located at 447 nm in cell-free extracts, but sometimes lower. Cell fractionation revealed that SCCYP51 was a soluble enzyme, being located in the supernatant following the 100000 ***g*** ultracentrifugation of cell homogenate. No CYP was detected in the membranes following heterologous expression.

Purification of recombinant SCCYP51

SCCYP51 was purified to homogeneity from *E. coli* supernatant following two passages through a Ni^{2+}-NTA affinity column. Subsequently, the recombinant protein was dialysed thoroughly against buffer A to remove excess salts and concentrated by ultrafiltration. The specific content of purified SCCYP51 was approx. 17.5 nmol/mg of protein and a single homogeneous band was observed on SDS/PAGE at approx. 52 kDa (Figure 1). The oxidized absolute spectrum of the purified SCCYP51 showed a Soret band at 417 nm and α- and β-bands at 567 and 534 nm, whereas 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 447 nm (Figure 2, upper panel).

Spectral characterization of recombinant SCCYP51

The purified recombinant SCCYP51 produced a type I spectrum with a peak at 390 nm and a trough at 422 nm, using both lanosterol and 24-MDL as substrates (Figure 2, lower panel). A type I-binding spectrum is indicative of shifting to a high-spin state on binding to sterol by substrate-free CYP where the associated haem is in a low-spin state [26].

Catalytic characterization of SCCYP51

Reconstitution of recombinant SCCYP51 with recombinant *E. coli* flavodoxin and flavodoxin reductase was carried out using both lanosterol and 24-MDL as substrates. After 20 min incubation, the reconstitution reaction mixture was extracted using ethanolic KOH and the sterols partitioned into hexane, silylated and analysed by GC–MS. Following reconstitution with 24-MDL, the extracted reaction mixture contained two principal sterols (Figure 3, top left and top right panels). The compound with a retention time of 42 min was silylated 24-MDL with a molecular ion at 512 m/z (Figure 3, middle panel). A compound, whose formation was dependent on the presence of CYP, had a retention time of 34 min and a molecular ion at 498 m/z (Figure 3, bottom panel). This compound was identified as the silylated 14α-demethylated 24-methylene-4α-methyl-5α-ergosta-8,14,24(28)-trien-3β-ol. In control sterol 14α-demethylation experiments, the fragmentation pattern of the molecular ion of the silylated product catalysed by SCCYP51 was identical with the MS-fragmentation pattern observed for the 14α-demethylated product from 24-MDL generated in a purified reconstituted *Candida albicans* CYP51/NADPH/CYP reductase enzyme system [27]. The activity was calculated to be 0.15 nmol of demethylated product formed/min per nmol of SCCYP51 for 24-MDL,

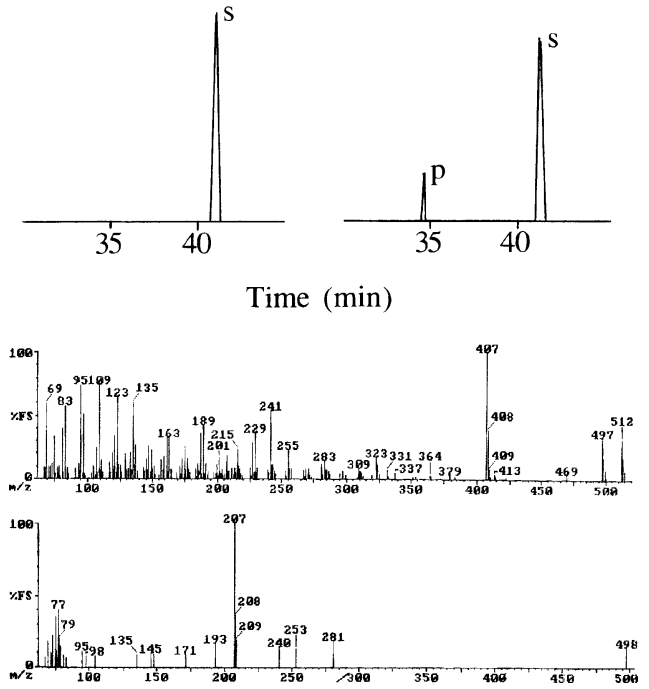

Figure 3 The catalytic properties of reconstituted SCCYP51, as analysed by GC–MS, using 24-MDL as a substrate

Recombinant SCCYP51 was reconstituted with flavodoxin and flavodoxin reductase. Sterols were extracted into hexane, silylated and subjected to GC–MS analysis. Top, left panel: GC profile of silylated 24-MDL (s) showing a retention time of 42 min. Top, right panel: GC profile of a reconstitution experiment demonstrating the metabolism of 24-MDL by recombinant SCCYP51. The demethylated product (p) has a retention time of 34 min. Middle and bottom panels: mass spectrum of silylated 24-MDL showing the molecular ion of *m/z* 512 (middle panel) and the mass spectrum of the demethylated silylated product formed from 24-MDL having a molecular ion of *m/z* 498 (bottom panel).

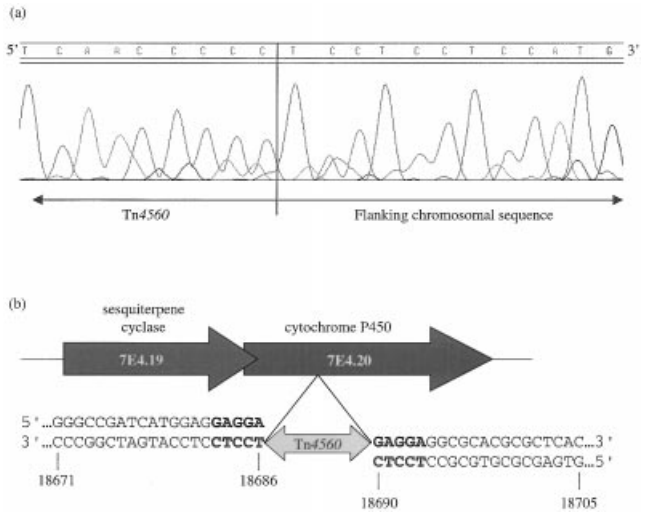

Figure 4 Transposon position data within *SSCYP51* in the mutant strain

(**a**) DNA sequence data on the Tn*4560* transposon insertion into *CYP51*. (**b**) A schematic representation indicating the location of the insertion into *CYP51*. Transposition of Tn*4560* generated a 5 bp direct repeat of the target site (indicated in bold). The location of the ORF for a putative sesquiterpene cyclase gene (7E4.19) relative to the CYP51 ORF (7E4.20) is also shown.

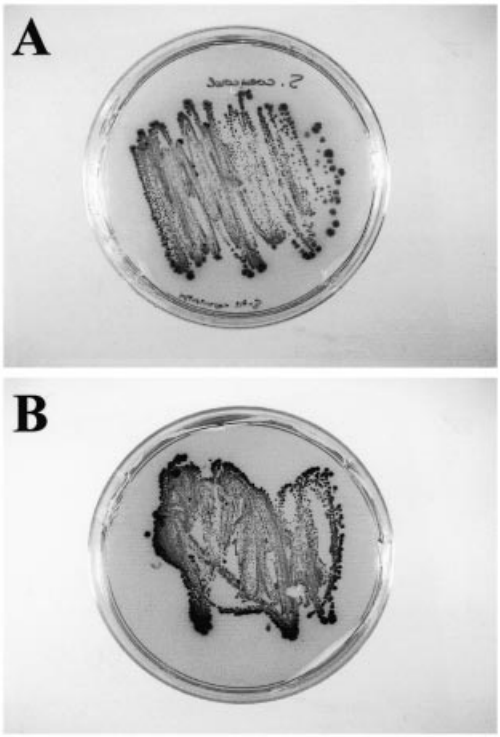

Figure 5 *SSCYP51* wild-type (A) and knockout strain (B)

An *S. coelicolor* A3(2) mutant containing a transposon insertion in the *CYP51* gene, which will abolish synthesis of the functional haemoprotein, was isolated as a viable strain, the first time a CYP51 has been identified as non-essential. No product was detected in non-saponifiable cell extracts of the parent *S. coelicolor* A3(2) strain nor were any detected in the lipid profile observed with the mutant.

but no metabolism was detected in the absence of CYP51 or NADPH. No demethylated product was observed following reconstitution of SCCYP51 with lanosterol at a concentration equivalent to that of 24-MDL (results not shown) and that used in other CYP51 studies (e.g. [27]).

SSCYP51 knockout and sterol profile

A transposon mutant library of *S. coelicolor* A3(2) containing 1×10^5 random insertions was screened for mutants containing a transposon insertion in CYP51 amongst a number of other target genes. A mutant was identified by PCR where the site of transposon gene insertion was 5′ to the *CYP51* sequence encoding the haem-binding domain. The co-ordinates of the *CYP51* gene are 18278–19663 in cosmid SC7E4. Using a transposon-specific primer for DNA sequencing, the Tn*4560*–chromosome junction was clear (Figure 4a). The 140 nucleotides of flanking chromosomal sequence used in a blastn search against *S. coelicolor* cosmid sequences (http://www.sanger.ac.uk/Projects/S_coelicolor/blast_server.shtml) gave 99% identity with *CYP51* on cosmid 7E4. Transposition of Tn*4560* generated a 5 bp direct repeat of the target site (indicated in bold in Figure 4b) and co-ordinates of which on the cosmid are 18686–18690 (Figure 4b). The location of the transposon event within the *CYP51* gene was 408 bp from the 5′-end and 973 bp from the 3′-end of the gene, i.e. 5′ to that region encoding the haem-binding domain. The haem-binding domain is located from ^{403}FSAGKRK**C**PS^{412} (where single-letter amino-acid notation has been used and the cysteine residue is shown in bold). As this domain is essential for any CYP catalytic activity, no functional CYP51 was produced

in the mutant. The fact that a CYP51 mutant was represented in a transposon mutant library was rather surprising given that CYP51 function in eukaryotes, such as yeast, is essential, and indicated that CYP51 is non-essential in *S. coelicolor* A3(2) (Figure 5). The presence of CYP51 in *S. coelicolor* A3(2) led us to investigate the sterol profile of both the parent and mutant strains. Using non-saponifiable cell extracts from 2 litres of stationary-phase cultures, no sterol product was observed in non-saponifiable extracts of cells grown in defined minimal medium (results not shown).

DISCUSSION

CYPs have been extensively studied due to their involvement in drug (xenobiotic) metabolism, carcinogen activation and both primary and secondary metabolism [28,29]. CYPs are found in some, but not all (*E. coli* has no CYPs), prokaryote species. So far, actinomycetes appear to have the greatest numbers of CYPs among bacteria with 20 genes identified in the *M. tuberculosis* genome (although interestingly only one was identified in the genome of *M. leprae* [30]). The annotation of the genome sequence of *S. coelicolor* A3(2) revealed 18 putative CYPs. To date, the identity of these as CYPs has only been confirmed by heterologous expression and spectrophotometric characterization (D. C. Lamb and S. L. Kelly, unpublished work).

Of these *S. coelicolor* A3(2) CYP genes, one resembled CYP51 and we demonstrate in the present study that it was indeed a sterol 14α-demethylase. Bloch [1] proposed the biosynthesis of cholesterol from lanosterol, and Alexander et al. [5] showed CYP was involved in the 14α-demethylation of lanosterol. Alexander

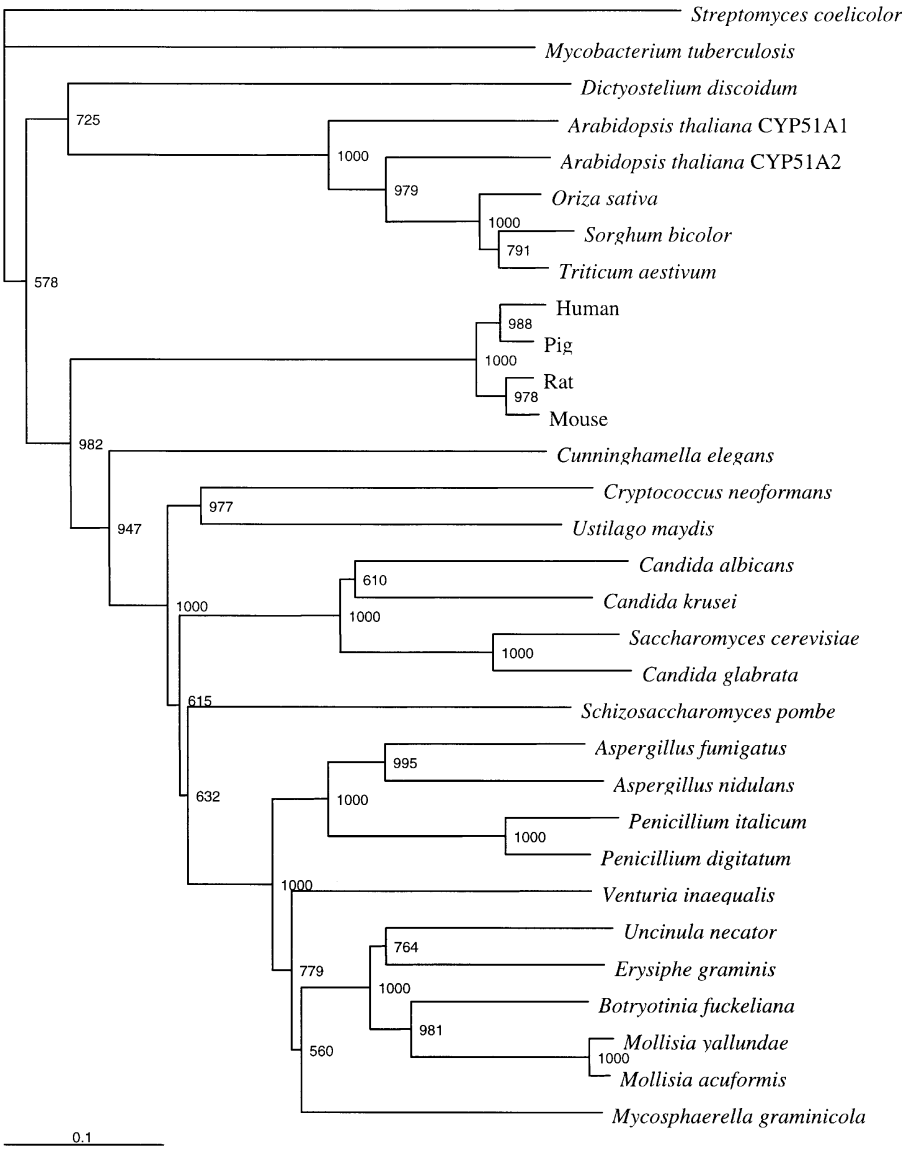

Figure 6 Most probable phylogenetic tree of the CYP51 family

Sequences published in GenBank® were aligned and trees were calculated using ClustalIX (obtained from http://www-igbmc.u-strasbg.fr). Numbers indicate the bootstrap probability values (1000 resamplings) of observing the branch topology shown. Trees were constructed using TreeView (obtained from http://taxonomy.zoology.gla.ac.uk/rod/rod/html). The bar in the lower left corner represents 0.1 amino acid substitution per amino acid for the branch length.

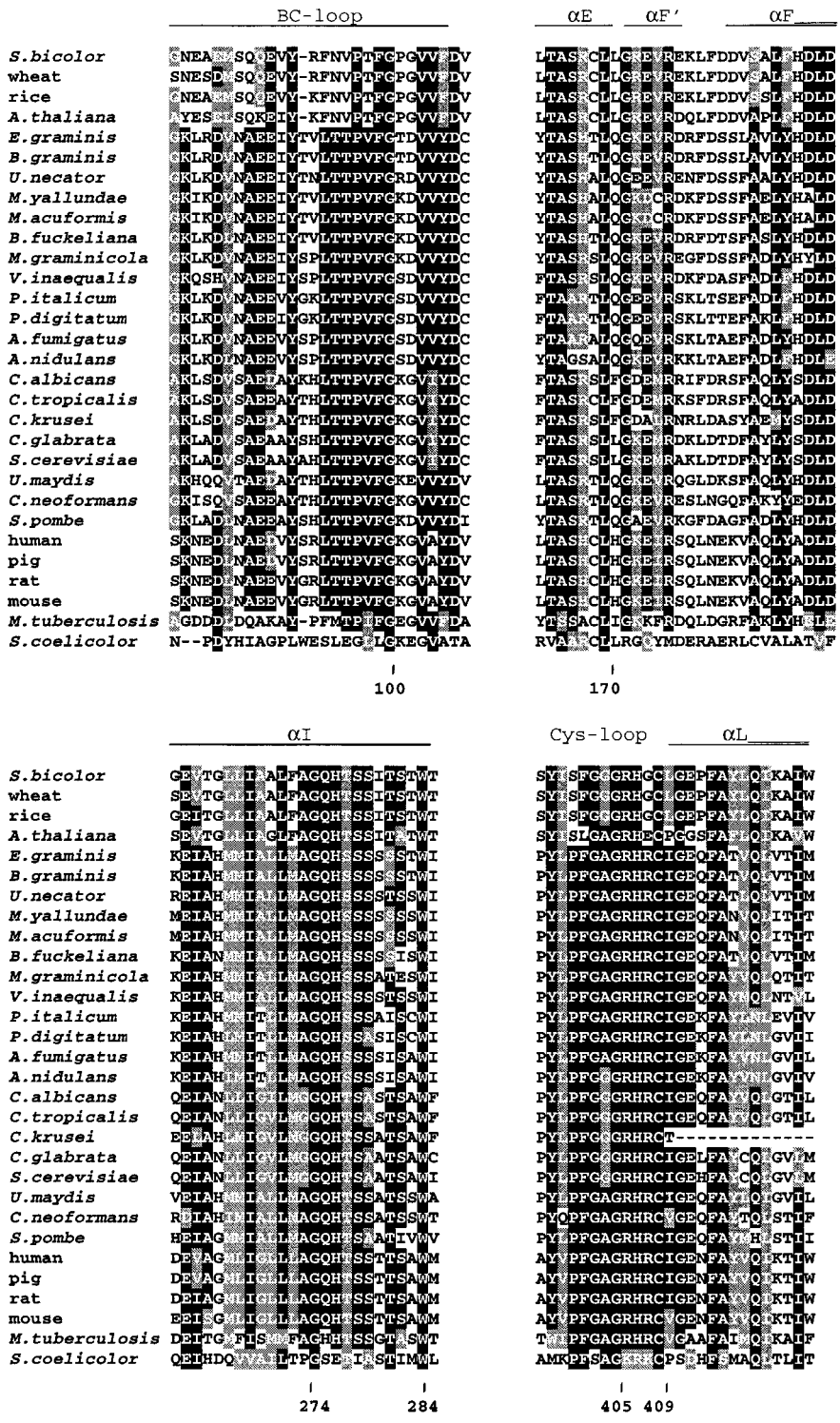

Figure 7 Four fragments of multiple sequence alignment between CYP51 family members and SCCYP51 represent regions with highest content of strictly conserved residues in CYP51

Positions that retain the same residue in SCCYP51 are indicated with black shading. Positions with amino acid substitutions are highlighted with grey shading. Helices (α) are assigned and residues are numbered according to CYP51 from *M. tuberculosis*. The loop between helices B and C (BC-loop) and the cysteine-pocket region (Cys-loop) are also indicated.

and co-workers [6] showed this mono-oxygenase demethylated sterols via sequential formation of alcohol, aldehyde and then release of formic acid. Subsequently, eukaryotic CYP51s have been investigated extensively and gene sequences, initially from *Saccharomyces cerevisiae* [31], indicated that they were the first CYP gene family uncovered in different kingdoms of life [32]. The post-genomic investigation of prokaryotic organisms revealed CYP51 to be also present in this kingdom [33].

The alignment of SCCYP51 in a phylogenetic tree of CYP51s shows that it clusters with *M. tuberculosis* and is closest to the eukaryotic CYP51s of *Dictyostelium discoidum* and the known plant sequences (Figure 6). Interestingly, metabolism of the non-C-24 alkylated sterol, lanosterol, was not detected in the present study and poor metabolism was observed in *M. tuberculosis* [17]. Only plant CYP51 enzymes have shown discrimination involving a lack of lanosterol metabolism [34,35]. The presence of this gene product is apparently not related to sterol biosynthesis as no such products were observed in our cultures of *Streptomyces*. Did the ancestral organism of *Streptomyces* and mycobacteria carry out sterol biosynthesis? Perhaps so, since *M. smegmatis* can synthesize non-saponifiable lipid [16]. However, no such compounds were found in *S. coelicolor* A3(2) and, so far, no ORFs in the genome are predicted to encode proteins homologous with genes of the sterol biosynthetic pathway except CYP51. We predict that *S. coelicolor* A3(2) lost such enzymes during evolution rather than *M. tuberculosis*, *M. smegmatis* and *M. bovis* gaining such homologues. *M. leprae* has lost CYP51 possibly because the organism has become an auxotroph obtaining relevant products from the host. Why then should *S. coelicolor* A3(2) retain CYP51 from an ancestral organism? Clearly a further role for CYP51 must be expected. Indeed, SC7E4.20 is downstream of, and overlapping with, SC7E4.19, a putative sesquiterpene cyclase. Thus it is entirely plausible that SC7E4.19 and SC7E4.20 have related functions and that CYP modifies the sesquiterpene cyclase product, possibly by hydroxylation (G.L. Challis, personal communication).

Although SCCYP51 has 14α-demethylase activity, comparative analysis of primary sequences of SCCYP51 and known CYP51s shows significant divergence in the functionally important regions, including the B′ helix and the loop between helices B and C [substrate recognition site-1 ('SRS-1')], the I helix [substrate recognition site-2 ('SRS-2')], the cysteine-pocket region, and the junction between E and F′ helices (Figure 7). CYP51s from different organisms show approx. 30% amino acid sequence identity. However, SCCYP51 is only 24% identical with *M. tuberculosis* CYP51. The alignment of 29 CYP51 sequences available to date in databases reveals that 32 residues are strictly conserved in the CYP51 family. Three of these residues, Glu^{330}, Arg^{333} and Cys^{410} (the haem ligand), are conserved throughout the whole CYP superfamily. Secondly, two proline and nine glycine residues distributed throughout the protein sequence are probably conserved, due to playing roles in the maintenance of the general protein architecture. The third, and the most interesting group of strictly conserved residues, are clustered in regions of functional importance related to catalytic activity. Together with the conserved proline and glycine residues, they create the sequence pattern characteristic for the CYP51 family. SCCYP51, when included in alignment with the 29 CYP51 family members noted above, reveals only 13 of the strictly conserved residues shared with other members of the family. Almost all of these are either conserved throughout the whole CYP superfamily or play a role in maintenance of the general protein architecture (one proline and six glycine residues). The characteristic CYP51 sequence pattern was not found in SCCYP51. Thus we conclude that, despite having sterol 14α-demethylase activity, SCCYP51 can hardly be considered as a conventional member of the CYP51 family. Perhaps SCCYP51 is the first member of a second CYP51 subfamily, CYP51B. Although SCCYP51 has retained 14α-demethylase activity, it must serve another function in *S. coelicolor* A3(2), a function which is not essential. The gene product of the *S. coelicolor* A3(2) ORF SC7E4.20 (possibly SCCYP51B) may be an extremely interesting model for studying CYP evolution.

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