# 13

# The Diversity and Importance of Microbial Cytochromes P450

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The cytochromes P450 (CYPs) of microbes are enormously diverse as revealed in discoveries from the era of molecular biology and as subsequently found in genomic investigations. One percent of the genes of a microbe can encode CYPs, but in stark contrast most bacteria studied so far can survive without CYPs. Microbial eukaryotes usually have at least one CYP, due to the essential requirement of most to synthesize sterol involving CYP51, sterol 14α-demethylase. The roles of the vast majority of microbial CYPs remain to be elucidated, but many already have important fundamental roles in nature, and others are important for biotechnological purposes. Some others have, of course, provided facile models for understanding CYP structure and activity, such as CYP101 ($P450_{CAM}$) of *Pseudomonas putida* and CYP102A1 ($P450_{BM-3}$) of *Bacillus megaterium*. The purpose of this chapter is to provide an outline of the important biomedical and environmental roles of the microbial CYPs, including many which were unsuspected when the respective microorganisms were originally studied. This includes involvement of CYP in some of the earliest metabolic alterations in the production of penicillin, some of the early biosynthetic steps allowing the production of corticosteroids, and the first application of therapeutic CYP inhibitors, the azole antifungal agents. Current and future applications involving microbial CYPs are manifestly clear, ranging from new therapeutics to biotransformations and bioremediation.

## 1. Introduction to Microbial CYPs

The discovery of cytochromes P450 in mammalian tissues rich with these proteins, such as liver and the adrenal gland, resulted in intense scrutiny of their roles in xenobiotic metabolism and endogenous functions[1–3]. Following their discovery came the realization that mammalian proteins required electron donor systems for activity, either NADPH-cytochrome P450 reductase (CPR) or adrenodoxin and adrenodoxin reductase in the endoplasmic reticulum or the mitochondria respectively[4, 5]. Protein biochemistry and molecular biology revealed the multiplicity of CYP forms and mammalian genomes exhibited CYP diversity.

The microbial CYP systems were studied at the same time, with yeast CYP being reported by Lindenmeyer and Smith (1964) and bacterial CYP by Appleby (1967)[6, 7]. The systems were viewed as models and this was true especially for a CYP from *P. putida* called $P450_{CAM}$ (CYP101) that allowed this bacterium to grow on camphor as a carbon source[8–12]. In pioneering work from the

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Gunsalus laboratory, the $P450_{CAM}$ system allowed biochemical and biophysical investigation of the CYP catalytic cycle as well as of the genetics of a bacterial catabolic plasmid. This typical CYP system was found to require a ferredoxin and ferredoxin reductase for catalytic activity, unlike the model for eukaryote CYPs, CYP102A1 or $P450_{BM-3}$, which was discovered in the Fulco laboratory and consisted of a fusion polypeptide containing CYP and reductase domains[13].

Studies in yeast have revealed deep insights into eukaryotic processes, and this is also true in studies on CYPs, where the first microbial CYP cloned was found to undertake an ancestral role in the superfamily. That is, CYP51 is needed for sterol biosynthesis and is found in plants, fungi, protists, animals, and some bacteria[14].

The nomenclature for CYPs is based on amino acid identity with 40% identity and above needed to place CYPs in the same family and more than 55% to place them in the same subfamily[15]. These rules can be relaxed, as is the case for CYP51s that can fall below 40% identity, if the CYPs undertake the same function. For microbial eukaryotes, the family numbers 51–69 and 501–699 are available and at the time of writing, numbers up to CYP553 are listed, but each genome reveals many more and many are not yet assigned. Bacterial CYP family numbers are initiated at CYP101 and a similar expanding scenario can be envisaged with more and more genomes.

In this chapter, we will outline historical perspectives on the discovery and importance of CYPs in biotechnology before going on to describe the diversity of functions and activities associated with microbial CYPs. The coverage is relatively extensive and is illustrative of the field, but with so many CYPs now revealed it is impossible to discuss each one individually. Obviously many of the CYPs that remain orphan in function today will emerge as being important in future studies.

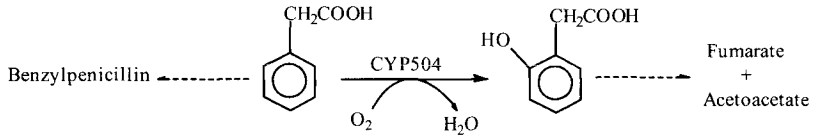
Microbial science is generally reported to begin with the fermentation of yeast observed by Pasteur, and although yeast CYP is not a cytochrome involved in respiration, it does contribute to the osmotic robustness of the microorganism, including ethanol tolerance, through the synthesis of ergosterol. This product requires CYP51 to remove the C14-methyl group of the precursor as well as a second CYP, CYP61, to undertake C22-desaturation[16]. Ergosterol in yeast has been used to produce vitamin $D_2$, although this has been uneconomical recently, but manipulation of this pathway has allowed production in whole-cells of hydrocortisone[17].

The modern era of biotechnology began with the discovery of antibiotics and the steps taken to improve yield. We now realize that streptomycetes contain many CYPs for drug (secondary metabolism) synthesis and this is discussed later in more detail, but *Penicillium chrysogenum* was the first utilized in antibiotic production. In early work following the pioneering studies, phenylacetate (precursor) feeding was found to elevate yields from fermentations and, of course, mutation and screening strategies increased the titer. Recently, the basis of genetic change in the Wisconsin strains revealed that a CYP mutation produced increased penicillin yield at the beginning of the genesis of improved fungal strains[18]. The gene concerned, *PahA* encodes CYP504, a CYP also identified in *Aspergillus nidulans* that allows growth on phenylacetate[19]. A *CYP504* mutant containing the substitution L181F resulted in the reduced 2-hydroxylation of phenylacetate, and this mutation channeled the carbon flux away from the side-pathway and through into increased penicillin titer.

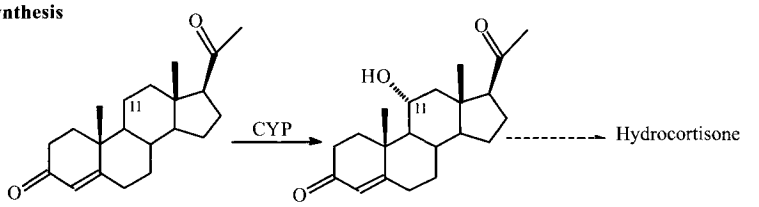
Parallel with developments after World War II in antibiotic production, the therapeutic value of corticosteroids was discovered. Interest during the early 1940s was based on rumors of experiments in performance enhancement of pilots in the Luftwaffe by corticosteroids[20]. The chemical synthesis route was inefficient and microbial hydroxylations by fungi were some of the first successful biotransformations for pharmaceutical production. 11α-Hydroxylation of a steroid was achieved by *Aspergillus ochraceous* and *Rhizopus niger*, and the 11β-hydroxylation achieved with other fungi such as *Cochliobolous lunatus*, allowing the production of cortisol. We now realize these conversions were achieved by fungal CYPs, although the genes concerned are not yet known.

A last example to note before moving onto describing the diversity of microbial CYPs and their importance, is provided by the azole antifungal compounds[21]. First developed for agriculture, where they are known as DMI compounds (demethylase inhibitors), these compounds have become central to antifungal therapy in the clinic

A. Mutation in CYP504 leads to overproduction of penicillin in *Penicillium chrysogenum*




B. Commercial application of P450 catalysed 11α-hydroxylation of progesterone in hydrocortisone biosynthesis




C. CYP51 and sterol biosynthesis, application to azole antifungal development

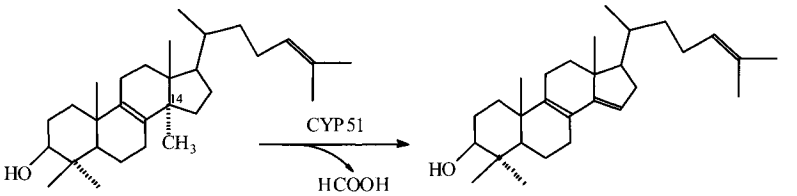



**Figure 13.1.** (A) The metabolism of phenylacetate by *Penicillium chrysogenum* by CYP504 is impeded during early strain improvement by mutation and selection increasing flux through to penicillin production; (B) The 11-hydroxylation of steroid by some filamentous fungi were among the first commercial biotransformations in the pharmaceutical industry allowing the production of corticosteroids, first 11α-hydroxylation by *Aspergillus* sp. and *Rhizopus* sp. and subsequently 11β-hydroxylation by *Curvularia* sp.; (C) Sterol C14-demethylation in fungi is the target of the antifungal azole compounds.

and new compounds continue to be evaluated. The mode of action in relation to CYP51 and the repercussions of that for the fungus only became clear after their development, but they were the first commercial CYP inhibitors. Some of the microbial CYP activities described here are shown in Figure 13.1.

## 2. Classes of Microbial CYPs

CYPs in bacteria are generally soluble proteins requiring ferredoxin and ferredoxin reductase for the two electrons needed in the CYP catalytic cycle, while CYPs in eukaryotic microbes are typically located in the endoplasmic reticulum with an associated NADPH-CPR providing the necessary reducing equivalents. As such, these are often called class I and class II, respectively (see also Chapter 4). Over the last 20 years other novel forms have been identified including the fusion protein CYP102A1 ($P450_{BM-3}$) identified in *Bacillus megaterium* by the Fulco laboratory[13, 22]. This CYP resembles the class II system with a flavoprotein reductase domain while CYP55 ($P450_{nor}$) is a stand-alone catalytic entity with an NADPH-binding site and a third class[23]. Over the last years, several new types have been identified which, in some cases, have been placed into classes. One response to this diversity is to have new classes for each new form and to not disrupt the assignments made already, and we have previously adopted that approach[24]. However, with the emergence of further forms of CYP fusion proteins, and the anticipation that more will arise, it is a suggestion here that classes should reflect novelty only in the method CYP reduction. In this way, CYP fusion proteins involving ferredoxin

and ferredoxin reductase would be subclasses of class I, while CYP fusions involving only flavoproteins would be subclasses of class II. Catalytically self-sufficient CYPs represent class III. Different subclasses can occur for the CYPs involved, but class I and class II would give the immediate impression of the type of electron transfer system concerned.

A number of new CYP fusion forms and a novel CYP operon have been cloned at the time of writing that represent new forms. The gene encoding CYP176A1 ($P450_{cin}$) was found in an operon with genes encoding a flavodoxin and flavodoxin reductase and so could represent an ancestor of the class II system. These would be placed as a class IIc after CYP102A1, class IIb[25]. The flavodoxin and flavodoxin reductase could have become fused in other class II systems. Also a novel CYP was identified from a *Rhodococcus* sp. containing a reductase domain at the C-terminus similar to dioxygenase reductase protein (containing flavin mononucleotide and NADH-binding domains) and a C-terminal ferredoxin center (2Fe2S) and the end of the polypeptide[26]. This catalytically self-sufficient enzyme will be of interest for biotechnological modifications and directed evolution studies, but as it contains a ferredoxin system, it could be placed within class Ib. A CYP51 has been observed in *Methylococcus capsulatus* fused to a C-terminal ferredoxin domain and unless this has a novel reductase partner, it can be considered a Class Ic form as it conforms to the ferredoxin/ferredoxin reductase model (Figure13.2)[27].

A further *CYP* gene has also been cloned that represents a new form. It confers a capability to metabolize the high explosive hexahydro-1,3,5-trinitro-1,3,5-triazine and has been identified in *Rhodococcus rhodochrous*, with a flavodoxin domain at its N-terminus, but appears also to need a ferredoxin reductase for activity, which may be encoded adjacent to the *CYP* gene[28]. As this gene contains a flavodoxin domain, this can be placed

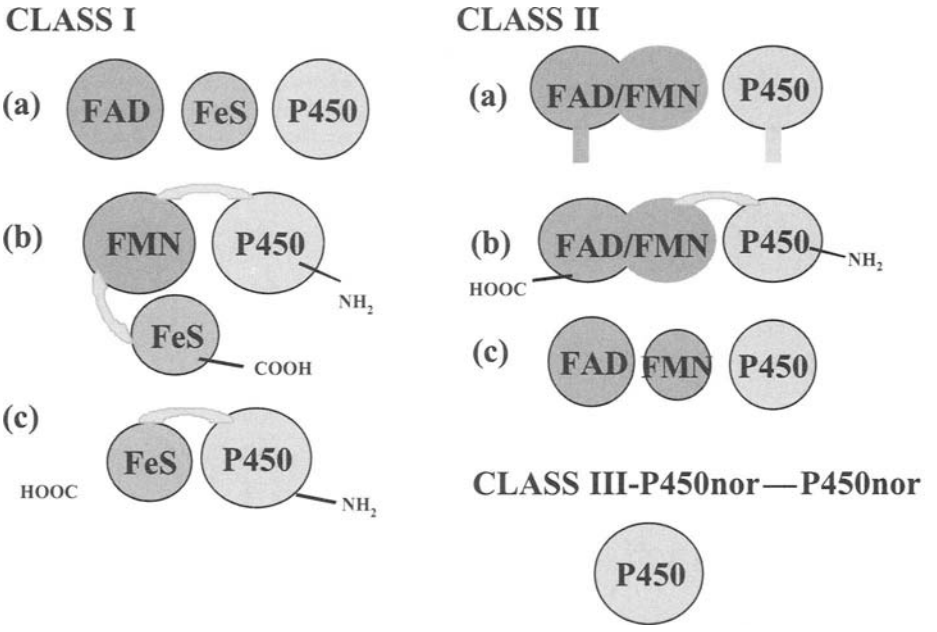



**Figure 13.2.** A proposal for a simplified classification of CYPs with reference to either use of a ferredoxin or alternative electron transport mechanism. Class Ia—typical bacterial system supported by ferredoxin and ferredoxin reductase, for example, CYP101; Class Ib—*Rhodococcus* sp. CYP fusion protein containing a ferredoxin domain[26]; Class Ic—*Methylococcus capsulatus* CYP51–ferredoxin fusion[27]. Class IIa—typical eukaryotic CYP/NADPH-cytochrome P450 reductase system; Class IIb—a fusion protein of a CYP and flavoprotein reductase, for example, $P450_{BM-3}$; Class IIc—$P450_{cin}$ containing separate flavodoxin and flavodoxin reductase partners[25]. Class III—stand-alone functional CYPs, for example, $P450_{nor}$[23].

with the Class II CYP systems if substantiated by protein studies. Another CYP has been detected in *Ralston metallidurans* and has a C-terminal fusion to a phthalate family oxygenase reductase module, but appears to be similar to another fusion described above[26, 29].

## 3. Considering the Origins and Relatedness of Microbial CYPs

Clues to the basic functions and the origins of CYPs can be found among the microorganisms. Among eighteen archaebacterial genomes probed, five contained *CYP* genes. Similarly, about two thirds of proteobacterial genomes (28 among 90 genomes probed), such as *Escherichia coli*, contain no CYPs, indicating how nonessential CYPs are to basic metabolism. This correlates with the involvement of CYPs in mechanisms of deterrence and attraction through the production of secondary metabolites, and in detoxification, and it is the general view that these selective pressures have produced much of the observed CYP diversity[14, 30–32]. However, some bacteria contain many CYPs, including *Mycobacterium smegmatis* that contains approximately 39 *CYP* genes, that is, about 1% of all genes in this microorganism, together with additional *CYP* pseudogenes[33].

CYPs have a role in bacteria to enable growth on carbon sources in the environment, such as on camphor by *P. putida* containing CYP101, or to undertake secondary metabolism as part of the biochemical warfare between organisms. Many CYPs are found in the pathways synthesizing important therapeutic compounds and this is discussed later in this chapter. The number of vital endogenous steps that have evolved for bacterial CYP function is still very small; for instance, BioI in *Bacillus subtilis* is needed to produce biotin by a pathway distinct from other bacteria[34]. Some CYPs, among the numerous forms uncovered, must be anticipated to be involved in key areas of endogenous metabolism especially in the

**Table 13.1.** The Numbers of CYPs in Various Sequenced Microbial Genomes

| Microorganism | CYP complement (CYPome) |
|---|---|
| Prokaryotes (most have no CYPs) | |
| *Campylobacter jejuni* | 1 |
| *Bacillus halodurans* | 1 |
| *Methanosarcinia barkeri* | 1 |
| *Mycobacterium leprae* | 1 |
| *Halobacterium* species *NRC1* | 1 |
| *Sulfolobus tokodaii* | 1 |
| *Sinorhizobium meliloti* | 2 |
| *Agrobacterium tumafaciens* | 2 |
| *Pseudomonas aeruginosa* | 3 |
| *Deinococcus radiodurans* | 3 |
| *Bacillus subtilis* | 8 |
| *Mycobacterium bovis* | 18 |
| *Streptomyces coelicolor* | 18 |
| *Mycobacterium tuberculosis* | 20 |
| *Streptomyces avermitilis* | 33 |
| *Mycobacterium smegmatis* | 39 |
| Eukaryotes (usually have CYPs) | |
| *Schizosaccharomyces pombe* | 2 |
| *Saccharomyces cerevisiae* | 3 |
| *Candida albicans* | 12 |
| *Neurospora crassa* | 38 |
| *Phanerochaete chrysosporium* | >100 |

morphologically and developmentally complex organisms. This is an important area of investigation, as therapeutic CYP inhibitors need targets that are implicated in viability or pathogenicity. No such general CYP target exists across the bacteria. Table 13.1 shows some examples of the numbers of CYPs present in bacterial genomes. It is clear that the actinomycetes can be especially rich in CYPs and that, unlike prokaryotes, eukaryotic microorganisms usually contain at least a few CYPs, including CYP51 that is used for making sterols.

## 3.1. CYP51 and Evolution of the Superfamily

Work on yeast resulted in the cloning of the first microbial *CYP*, *CYP51*, encoding sterol 14-α-demethylase, in the Loper laboratory[35]. Orthologues of this CYP were later revealed to be present in animals[36, 37], and plants[38]. Many different CYP51s have now been identified and Figure 13.3 shows a phylogram of CYP51 sequences. Substrates differ slightly between the different kingdoms[39], especially in plants where the enzymes studied so far utilize the C4-methyl sterol obtusifoliol as the substrate and not the C4-dimethyl substrate (e.g., lanosterol) used in fungi and animals. CYP51 is found throughout eukaryotes, although some, for example, nematodes and insects obtain sterol from their diet. The primitive eukaryote *Giardia lamblia* also lacks *CYP* genes as judged by probing of its genome using conserved heme-binding motifs for CYP, but this remains unusual among eukaryote genomes that may require sterols for stabilizing membranes. In general, most eukaryote genomes searched to date have at least a few *CYP*s. The question arises: If sterol biosynthesis represents an early CYP function, when did it arise? Previous thoughts on what early function CYPs might have evolved to undertake have centered on their potential role in detoxifying oxygen[40]. Within sterol biosynthesis, squalene epoxidase is a non-P450 monooxygenase preceding CYP51 and it is probable that CYP51 conferred on primitive microorganisms a more robust membrane by producing sterols. An alternative CYP ancestor could easily have preceded CYP51, of course. Following squalene epoxidase, the next enzyme needed to make sterols is 2,3-oxidosqualene sterol cyclase (or lanosterol synthase). This is again a point of divergence in metabolism, with many plants and some protists synthesizing cycloartenol using the related cycloartenol synthase rather than lanosterol synthase, prior to producing obtusifoliol for CYP51 metabolism[41].

The original route to sterol biosynthesis is unclear as some protists synthesize lanosterol and many, like fungi, produce ergosterol as an end-product[42]. Recently, *Mycobacterium tuberculosis* was revealed to possess a CYP51 with sterol 14-demethylase activity[43], and this protein has been crystallized[44]. The true function of this protein remains to be clarified[33], as the earlier detection of mycobacterial sterols was probably the result of sequestration from medium. Other bacteria have been shown to contain sterols at relatively high concentration, as confirmed by purification and nuclear magnetic resonance (NMR) studies. The earliest of these is the methane utilizing proteobacterium *M. capsulatus* that synthesizes sterols via a lanosterol route[45], but not as far as ergosterol, sitosterol, or cholesterol. This organism contains a CYP51 and as such was the first proven bacterial sterol biosynthesis gene and protein studied[27]. The closest homologues to this CYP51 are among the mycobacteria, and then CYP170 of *Streptomyces coelicolor* and plant and protist CYP51 (Figure 13.3). More recently, another myxobacterium has been revealed to produce sterols and a cycloartenol synthase has been identified, although a CYP51 awaits identification from this bacterium[46]. We have also identified a lanosterol synthase and squalene epoxidase homologues at a locus in *M. capsulatus* (unpublished observation).

It seems reasonable to assume that sterol biosynthesis arose in gram-negative bacteria at least, and possibly in gram-positive bacteria, although the homologues here could have been the result of horizontal transfer. The need for several genes that are unlinked in order to make bacterial sterols seems to suggest that sequential horizontal transfer or independent evolution is unlikely, and that a sterol biosynthetic pathway may have evolved as a feature of prokaryotic ancestors of eukaryotes. There is a cycloartenol-type (plant-like) pathway, as well as a lanosterol-type (fungal/animal-like) pathway, in different bacteria that provides an extra level of complexity to these considerations, as it might be expected that, if ancestral, a single

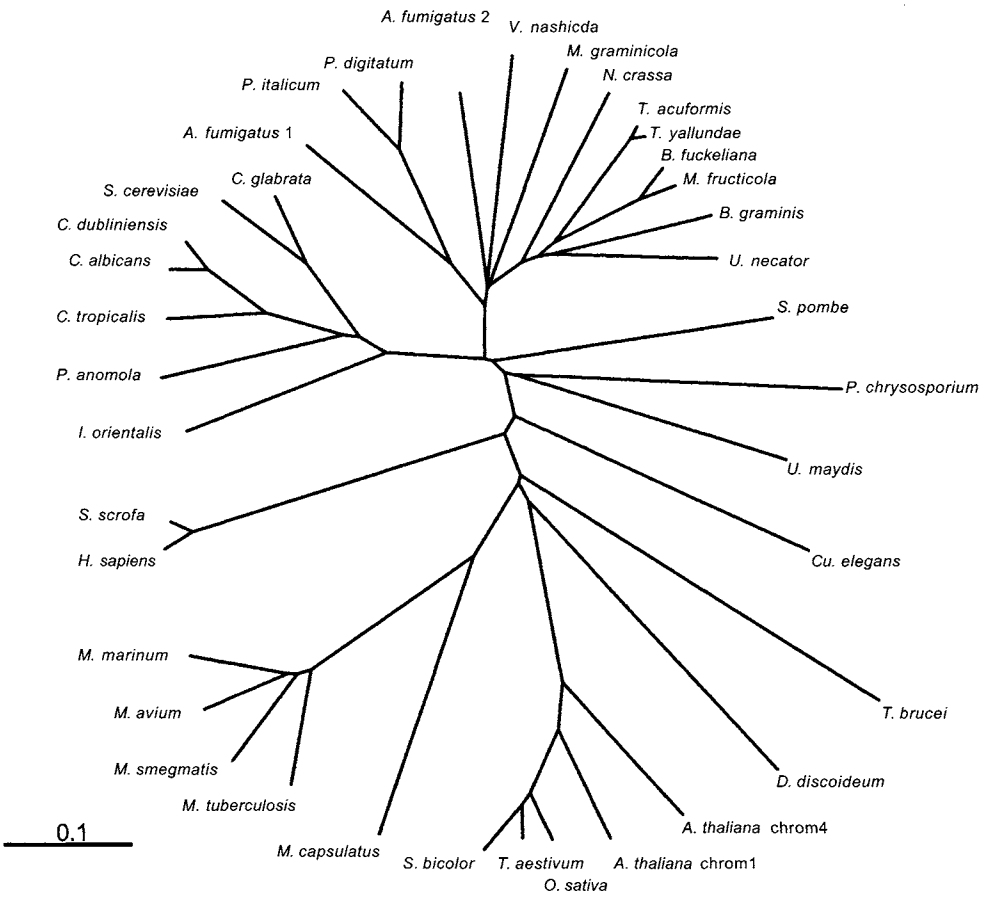



**Figure 13.3.** A phylogenetic tree containing sequences from bacterial, fungal, protist, plant, and animal CYP51s constructed using clustal X and Treeview 6. The sequences are *Aspergillus fumigatus* CYP51 1 (AF222068), *Aspergillus fumigatus* CYP51 2 (AF338660), *Botryotinia fuckeliana* (AF346594), *Blumeria graminis* (AF052515), *Candida albicans* (AB006856), *Candida dubliniensis* (AY034867), *Candida glabrata* (S75389), *Candida tropicalis* (M23673), *Cunninghammela elegans* (AF046863), *Issatchenkia orientalis* (S75391), *Mycosphearella graminicola* (AF263470), *Neurospora crassa* (http://drnelson.utmem.edu), *Penicillium italicum* (Z49750), *Phanerochaete chrysosporium* (http://drnelson.utmem.edu), *Pichia anomola* (AF019903), *Saccharomyces cerevisiae* (M18109), *Schizosaccharomyces pombe* (NC003424), *Tapesia acuformis* (AF208657), *Tapesia yallundae* (AF276662), *U. maydis* (Z48164), *Venturia nashicola* (AJ314649), *Dictyostelium discoideum* (http://drnelson.utmem.edu), *Trypanosoma brucei* (AF363026), *Arabidopsis thaliana* (chromosome 1 CYP51, AY05860), *Arabidopsis thaliana* (chromosome 4 CYP51, NC003071), *Oryza sativa* (AB25047), *Sorghum bicolor* (U74319), *Triticum aestivum* (AJ251798), *Homo sapiens* (U23942), *Sus scrofa* (AF198112), *Methylococcus capsulatus* (TIGR 414), *Mycobacterium avium* (TIGR 1764/3294), *Mycobacterium marinum* (Sanger mar388e06), *Mycobacterium smegmatis* (TIGR 1772/3269), and *Mycobacterium tuberculosis* (AL123456).

type of prokaryotic pathway would exist. Some clarification will emerge with more post-genomic studies.

The further evolution of CYPs must in some cases have included gene duplication events, including involving *CYP51* individually, besides genome duplication. Generally, it is accepted that pathways can evolve sequentially. The progressively more complex tailoring of sterols was selected for in the microbial era based on increased fitness of the organism, probably when exposed to physical parameters such as osmotic

stress. In fungi a further *CYP*, *CYP61*, evolved to perform the C22-desaturation of sterols and this may have evolved after a gene duplication of *CYP51*. The two gene families today have very little sequence identity (approx. 30%). The fungal sterol, ergosterol, is also found in protists such as trypanosomes and algae such as *Chlamydomonas reinhardtii*[47, 48], and so a CYP61 may be encountered elsewhere as proposed earlier[49]. Stigmasterol production in plants requires a C22-desaturation and yet no CYP61 was observed in *Arabidopsis thaliana*. The closest amino acid identity of a plant CYP to CYP61 was for CYP710, and no CYP61 has yet been seen in the green alga *C. reinhardtii*, which also makes ergosterol. It may be that other CYP families undertaking sterol C22-desaturation will need to be reclassified as CYP61 due to common function. Obviously, higher organisms and those that have homeostasis exist in a different evolutionary scenario from single-celled microbes. This presumably resulted in selection for other sterols such as cholesterol in animals and the array of phytosterols in plants. In some cases, such as in nematodes and insects that obtain sterol from their diet, sterol biosynthesis has been lost, while most bacteria have never had this requirement.

## 3.2. Bacterial CYP51

The identification of sterols in bacteria was first achieved in 1971 for the gram-negative bacterium *M. capsulatus*, where the end-products reported are various C4-methyl and desmethyl products[45]. A *CYP51* was identified in the genome of this bacterium with homology to other *CYP51*s and represented a new form with a (3Fe4S) ferredoxin domain fused at the C-terminus of the CYP domain via an alanine rich linker[27]. This 62-kDa protein was expressed and purified from *E. coli* and metabolized lanosterol (0.24 nmol/min/nmol protein) on addition of a ferredoxin reductase and NADPH. Other homologues of eukaryotic sterol biosynthesis, for example, lanosterol synthase and squalene epoxidase, are evident in this genome, although no genes are detected so far for the formation of end-product of 4-methyl and 4-desmethyl sterols. With the presence of many prokaryotic genomes it has become obvious that while some contain open-reading frames with considerable identity to sterol biosynthesis proteins, they are not all part of a sterol biosynthetic apparatus.

Another *CYP51*-like gene was identified in the emerging genome of *S. coelicolor* A3(2) and the protein had sterol 14-demethylase activity, but was not essential when the gene was knocked out and no sterols are made by this microbe[50]. This gene has been assigned to a different family (*CYP170A1*) and the protein shares 23 of the conserved amino acids across CYP51s, whereas the other CYP51s share approximately 40 amino acids.

One of the first important bacterial pathogens sequenced, *M. tuberculosis*, was found to contain 20 CYPs, one of which was identified as a CYP51. The hypothesis of azole inhibition of this CYP51, and parallels to antifungal activity with inhibition of fungal CYP51, stimulated the examination of the sensitivity among mycobacteria to known antifungal drugs. It was shown in *M. smegmatis* that potent activity of azole antifungals existed, except for fluconazole, but particularly for the topical agents[51], and this was also found in another study[52]. The latter report and a separate assessment of *Mycobacterium bovis* BCG sensitivity[14], showed less sensitivity in this slow-growing bacterium than in the fast-growing species, where the minimum inhibitory and bactericidal concentrations of miconazole were less than 2 μg/ml. Our further unpublished work indicates that the sensitivity of *M. tuberculosis* resembles that of *M. bovis* BCG, but other pathogenic species causing skin infections, *Mycobacterium chelonei* and *Mycobacterium fortuitum*, are sensitive and may be treatable with current topical antifungals (Table 13.2). To develop potent azole-type inhibitors as antimycobacterial agents will require screening other compounds, and it may well be that CYP51 is not the target, although both *M. tuberculosis* and *M. smegmatis* CYP51 bind azoles with high affinity[33, 52]. Indeed another *M. tuberculosis* CYP, CYP121, also shows high affinity binding for azole compounds[53]. Other CYPs from *M. tuberculosis*, such as CYP125, bind antifungal azoles poorly (unpublished observation). Further work in this area is needed to establish the mode of action, but while *M. smegmatis* is sensitive to azole compounds it contains no CYP121. It does, however, contain a strong homologue of the sole *Mycobacterium leprae* CYP, CYP164A1, with which the *M. smegmatis* CYP164A2 has 60% identity. No CYP family is

**Table 13.2.** Sensitivity of Mycobacteria to Azole Compounds Plus Amphotericin B Compared to Against *C. albicans*

| | Minimum Inhibitory Concentration (μg/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Antifungal | *C. albicans* 505 | *M. smegmatis* 700084 | *M. smegmatis* 13116 | *M. fortuitum* 01/2341 | *M. chelonei* *LRT* 6680 | *M. bovis* BCG | *M. tuberculosis* 1 | *M. tuberculosis* 2 |
| Clotrimazole | <2 | <2 | <2 | 4 | <2 | 16 | 64 | 64 |
| Econazole | <2 | <2 | <2 | 8 | <2 | 8 | 32 | 32 |
| Fluconazole | 8 | >256 | >256 | >256 | >256 | >256 | >64 | >64 |
| Ketoconazole | <2 | 16 | 16 | >256 | 8 | 32 | — | — |
| Itraconazole | — | — | — | — | — | — | >64 | >64 |
| Imidazole | >256 | >256 | >256 | ND | ND | ND | — | — |
| Miconazole | 4 | <22 | <2 | 8 | <2 | 8 | 32 | 32 |
| Tebuconazole | 4 | 32 | 32 | 64 | ND | ND | — | — |
| Amphotericin B | <2 | >256 | >256 | ND | ND | ND | — | — |

*Notes*: Clotrimazole, econazole, and miconazole, showed most efficacy against *M. smegmatis*[51], and also against pathogenic mycobacteria such as *M. chelonei* and *M. fortuitum* that cause skin infections resistant to treatment with standard antimycobacterial agents. Less activity was seen with these compounds against the slow growing species *M. bovis* BCG and against two strains of *Mycobacterium tuberculosis*. Other azoles show lower activity and fluconazole was not active. The ND denote no assay information was available.

present in all mycobacteria to present a common antimycobacterial target, although many are present in all so far known except *M. leprae* (Table 13.3).

The genome sequences of various mycobacteria have been completed and it became apparent that *CYP51* was part of a putative hexacistronic operon that was conserved across mycobacteria except for *M. leprae*, in which no gene or pseudogene exists for *CYP51* due to divergent evolution and massive gene decay[54]. One striking similarity is the close homology between the CYP51 of mycobacteria and that from *M. capsulatus*. This is also true for the ferredoxin gene that lies downstream of *CYP51* in mycobacteria, but is fused to the CYP51 domain in *M. capsulatus*. Possibly the *CYP51* in mycobacteria was transferred by horizontal transfer from an ancestor of *M. capsulatus*, where it has possibly been recruited to a new function linked to the other genes in that putative operon/gene cluster. Also included among the genes is another *CYP*, *CYP123*. If the CYP51 performs a diffferent endogenous role from other CYP51s, this would require reclassification, possibly as a CYP170 as with the *CYP51*-like gene of *S. coelicolor*.

## 4. Archetypal Bacterial CYPs

Table 13.1 shows a list of some bacteria with the number of CYPs associated with their genomes to date. Most striking are the number and diversity seen among actinomycetes such as streptomycetes and mycobacteria, although some actinomycetes, such as *Corynebacterium diptheriae*, have no CYPs. Much of the CYP diversity is likely to be due to their role in secondary metabolism, as is true in the filamentous gram-negative

**Table 13.3.** The CYPome of *M. tuberculosis* Compared to Another Strain with Genomic Information and the Presence or Absence of these Forms in *M. bovis*, *M. avium*, *M. smegmatis*.

| CYP number | Gene | H37Rv CDC1441,210 | *M. bovis* | *M. avium* | *M. smegmatis* | *M. leprae* |
|---|---|---|---|---|---|---|
| *CYP51* | *Rv0754c* | + | + | + | + | |
| *CYP121* | *Rv2276* | + | + | | | |
| *CYP123* | *Rv0766c* | + | + | + | + | |
| *CYP124* | *Rv2266* | + | + | + | + | *ML1787* |
| *CYP125* | *Rv3545c* | + | + | + | + | *ML2024* |
| *CYP126* | *Rv0778* | + | + | + | + | *ML2229* |
| *CYP128* | *Rv2268c* | + | + | | | |
| *CYP130* | *Rv1256c* | + | | + | + | *ML1102* |
| *CYP132* | *Rv1394c* | + | + | | | |
| *CYP135A1* | *Rv0357c* | + | + | | | |
| *CYP135B1* | *Rv0568* | + | + | | + | |
| *CYP136* | *Rv3059* | + | + | + | + | *ML1742* |
| *CYP137* | *Rv3685c* | + | + | | | |
| *CYP138* | *Rv0136* | + | + | + | + | *ML2684* |
| *CYP139* | *Rv1666c* | + | + | + | | *ML1237* |
| *CYP140* | *Rv1880c* | + | + | + | + | *ML2033* |
| *CYP141* | *Rv3121* | + | | | | |
| *CYP142* | *Rv3518c* | + | + | + | + | |
| *CYP143* | *Rv1785c* | + | + | + | | *ML1542* |
| *CYP144* | *Rv1777* | + | + | + | + | *ML1185* |
| *CYP102* | | | | | | *ML0447* |
| *CYP102* | | | | | | *ML2159* |
| *CYP164* | | | | + | + | *ML2088* |

*Notes*: Also shown is whether a pseudogene for the *M. tuberculosis CYP* exists in *M. leprae*. *ML0447* and *ML2159* (*M. leprae* ORF identifier) are identical pseudogenes in *M. leprae* that resemble *S. coelicolor A3(2) CYP102B1* and *ML 2088* is the only putative functional CYP of *M. leprae* where another member of this family is only found in *M. smegmatis*. The TIGR databases were utilized here.

myxobacterium that produces the anticancer drug epothilone[55], and the numerous forms present in the rifamycin gene cluster of *Amycolatopsis mediterranei*[56]. Some examples of bacterial CYP reactions are shown in Figure 13.4.

Many of the bacterial forms are described by David Nelson in his central website for CYPs (http://drnelson.utmem.edu/nelsonhomepage.html), but others are not yet assigned CYP numbers. The archetypal bacterial CYP is of course $P450_{CAM}$, assigned as CYP101, obtained from the bacterium *P. putida* ATCC17453[8–11]. This enzyme catalyzes the 5-*exo* hydroxylation of camphor, part of the breakdown of this carbon source for growth. The *CYP101* operon contains the *CYP* (*camC*) together with class I electron donor partners, a putidaredoxin reductase (*camA*), and putidaredoxin (*camB*). A fourth gene *camD* encodes a 5-*exo* hydroxy camphor dehydrogenase and the operon *camDCAB* is controlled by a *camR* repressor. CYP101 still provides an archetypal system for investigations of the CYP catalytic cycle and was the first CYP structure that revealed the triangular prism shape of the proteins and the heme with its cysteinyl thiolate ligand (see Chapter 3)[57, 58].

Other bacterial CYPs also undertake the breakdown of carbon sources for microbial growth. For instance, CYP108A1 ($P450_{TERP}$) metabolizes terpineol[59], and CYP176A1 ($P450_{cin}$) can metabolize cineol[25], while others can metabolize pollutants such as thiocarbamate herbicides and atrazine, as illustrated by CYP116 from a *Rhodococcus* sp.[60]. The CYP105 family of streptomycetes especially is associated with a wide variety of xenobiotic

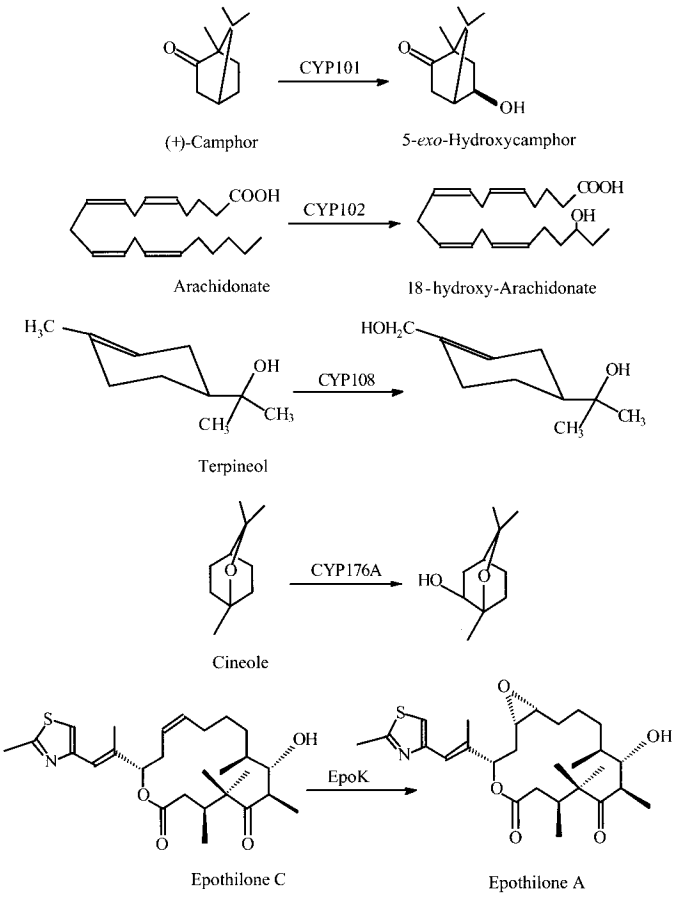



**Figure 13.4.** Examples of bacterial CYP reactions.

metabolism, although roles in pathways of secondary metabolism are also emerging[61].

As with all CYP activities, the substrates are largely lipophilic, and this is true of the other bacterial archetypal CYP, CYP102A1 ($P450_{BM-3}$), which as a class II system utilizing an FAD and FMN containing reductase domain, has provided a model for eukaryotic CYPs. This protein, containing both CYP and reductase domains in a soluble fusion protein that metabolizes various fatty acids, mostly at ω-2, was characterized in a series of publications from Fulco and colleagues during the 1980s[13, 62–64]. The rates of various reactions have been studied and this protein turns over substrate more efficiently than other CYPs utilizing separate redox proteins, with a rate of 17,000 $min^{-1}$ observed for arachidonate metabolism. Structural considerations for this protein are not the object of this chapter, but resolution of the structure of the CYP domain was a landmark in the field[65], and allowed numerous subsequent investigations using site-directed mutagenesis to probe the structure as well as the reductase domain[66–68].

Besides being present in proteobacteria, CYPs are also present in archaebacteria and some of these, CYP119 from *Sulfobolus solfataricus* and CYP175 from *Thermus thermophilus*, have had their structure solved[69, 70]. While these are interesting, the absence of known endogenous function precludes their discussion here. Thermophilic CYPs have been studied and we await functions, as well as CYP stucture/function for CYPs with activity at low temperatures that may have industrial uses.

## 5. Biodiversity of Bacterial CYPs and the Actinomycetes

The actinomycete bacteria encompass a wide range of species, including *Rhodococcus* spp., *Corynebacterium* spp., *Mycobacterium* spp., and *Streptomyces* spp., and represent important organisms for biotechnology in terms of enzymes, natural products, biotransformations, and bioremediation. Many are saprophytic, soil-inhabiting, gram-positive bacteria with a high G+C content, and some are also life-threatening human pathogens.

As mentioned earlier, many bacteria, including within the actinomycetes *C. diptheriae*, possess no CYPs, but the actinomycetes have revealed many genomes containing numerous CYPs. Mostly these are orphans with no known function, but included are new classes of CYPs from *Rhodococcus* described above, and from genome projects the diversity is surprising, with 20 *CYPs* in *M. tuberculosis*[71], 18 in *S. coelicolor*[72, 73], 33 in *S. avermitilis*[74], and 39 in *M. smegmatis*[33]. The genomes of streptomycetes are larger than the mycobacterial genomes with almost twice as many genes arranged on a linear chromosome. Thus, *S. coelicolor* contains 18 *CYPs* among approximately 7,825 open reading frames, that is, 0.2% of genes, while *M. smegmatis* has 39 out of approximately 3,800 genes (1% of genes). This latter proportion is similar to that observed in plants. Figure 13.5 shows a phylogenetic tree of the mycobacterial CYPomes of both *M. tuberculosis* and *M. smegmatis*.

The genomes of the mycobacteria and streptomycetes do not contain many CYP families in common. Both contain a *CYP51*-like *CYP*, and in *S. coelicolor* and *S. avermitilis* this *CYP* is called *CYP170A* and lies adjacent to a sesquiterpene cyclase that may well be of related function[50, 74]. One *CYP*, *CYP125*, was originally observed in *M. tuberculosis*, and recently a *CYP125* was also found in *S. avermitilis*.[74] *CYP105s* originally found in streptomycetes and associated with xenobiotic metabolism have also now been identified in *M. smegmatis* (Figure 13.5), as well as a *CYP107*[75–78]. The only other CYP family seen in both genera is a *CYP102* identified in *S. coelicolor* and *S. avermitilis*[73, 74], and as a pseudogene seen in the dramatic gene decay observed in *M. leprae* (Table 13.3).

### 5.1. Mycobacterial CYPs

The diversity of mycobacterial CYPs has been mentioned and trees relating the different CYP families of *M. tuberculosis* and *S. coelicolor* indicate they are quite different, reflecting the many hundreds of millions years since divergence from a common ancestor.[14, 73] A list of CYPs of *M. tuberculosis* is shown in Table 13.2, where the presence of these families in other mycobacteria is also shown. All *M. tuberculosis* CYPs conform to the expected conserved amino acids within the sequence of a CYP, including a conserved T

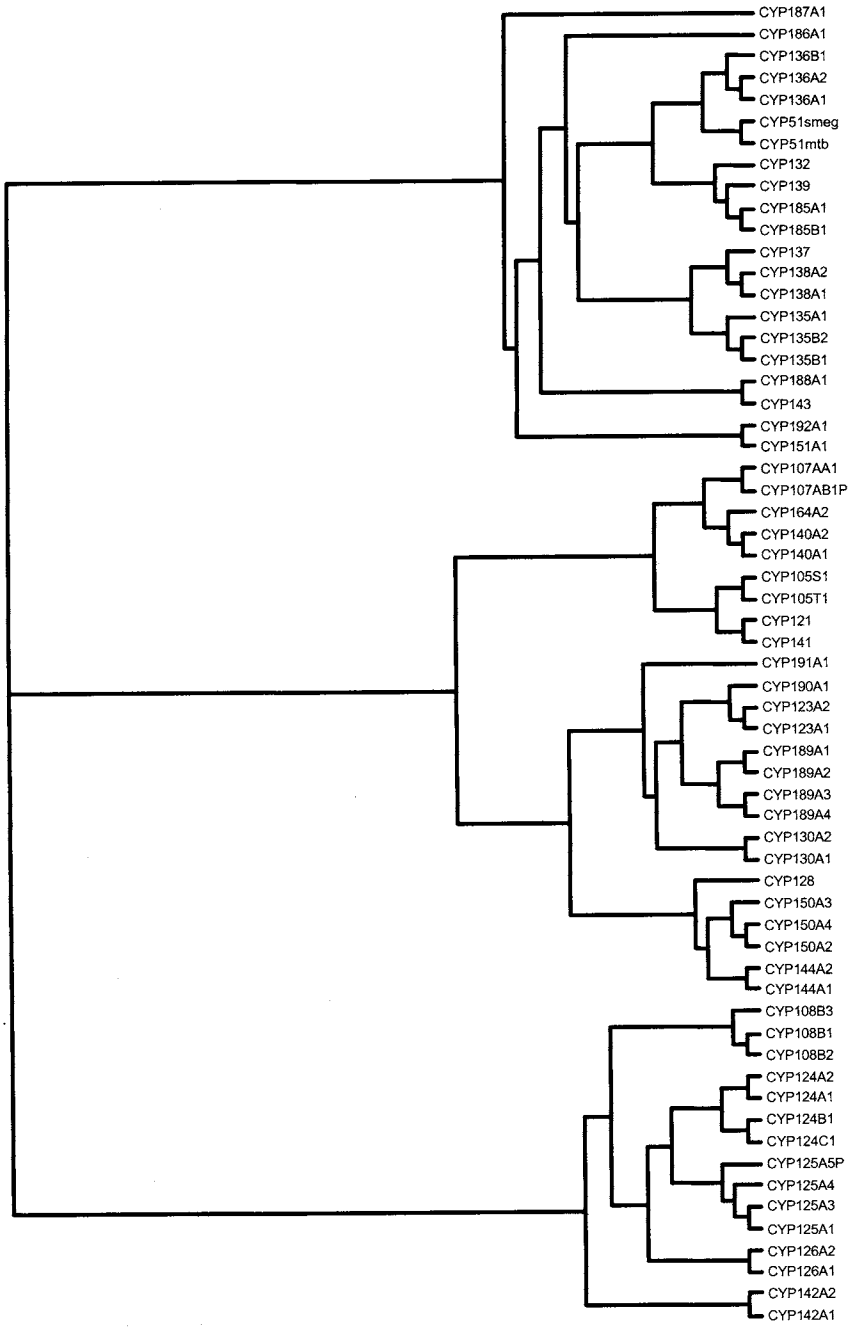



**Figure 13.5.** A phylogenetic tree of the CYPomes of *M. tuberculosis* and *M. smegmatis* using Clustal X and Treeview. Where present the *M. tuberculosis* CYP family member is designated A1. CYP families 121, 128, 132, 135A1, 137, 139, 141, and 143 of *M. tuberculosis* are missing in *M. smegmatis*, but a homologue of *M. leprae* CYP164A1 (CYP164A2) is included. New families of CYPs 186–192 were identified, new members of the CYP108 family and CYP105 and CYP107 family members are also found in streptomycetes. CYP125 showed two genes and a pseudogene (CYP125A3, A4, and A5P).

within the I-helix involved in oxygen-binding/electron transport, an EXXR motif in the K-helix, and a C-heme ligand in the C-terminal region.

Compared to the published sequence of the *M. tuberculosis* genome, *M. bovis*, although very closely related to *M. tuberculosis*, has only 18 *CYP*s, with *CYP130* being absent and *CYP141* being present as a pseudogene. The sequence of *M. leprae* showed massive gene decay as this organism moved toward parasitism, with only approximately 1,800 genes retained vs 3,800 in *M. tuberculosis*. Only 12 close homologues of the *M. tuberculosis CYP* complement were detected in *M. smegmatis* (Table 13.2), and this has the largest complement so far with 39 *CYP*s. When completed, the genomes of the related species *Mycobacterium avium* and *M. avium* ssp. *paratuberculosis* will have a similar number of *CYP*s, based on preliminary analysis of the data deposited at TIGR.

*CYP164A2*, the *M. smegmatis* homologue of *M. leprae ML2088* (*CYP164A1*), is 1,245 bp in length, encoding a predicted protein of 414 aa with a molecular weight of 44.9 kDa. By comparison, *CYP164A1* is 1,305 bp long and encodes a predicted protein of 434 aa with a molecular weight of 47.6 kDa. *M. smegmatis CYP162A2* is 60% identical (249/415) and 75% similar (313/415) in a 415 aa overlap with *CYP164A1* (BLASTP score e-130). Homology extends across all regions of the proteins, with only two gaps. In contrast, the closest *M. tuberculosis* homologue to CYP164A1, CYP140, shows only 38% identity (145/379) and 515 similarity (196/397), with 30 introduced gaps (BLASTP score 3e-58). The leprosy genome also contains a separate pseudogene of *M. tuberculosis CYP140* at locus *ML2033*, so these *CYP*s are likely to be functionally distinct.

The analysis of the CYP families present in *M. smegmatis*, reveal that, as expected, many new families covering *CYP186–192*, have been identified. The CYP family members in *M. tuberculosis CYP121*, *128*, *132*, *135A*, *137*, *139*, *141*, and *143* are not found in *M. smegmatis*. One CYP had already been identified before the *M. smegmatis* genome sequence, and this CYP was involved in morpholine utilization (*CYP151*)[79]. Interestingly, this soil microorganism has been associated with useful bioremediation properties and this may in some instances be associated with the CYP complement. Included in this CYPome are the first other members of the CYP108 family similar to $P450_{TERP}$ (CYP108A1)[59]. This may well reflect utilization of similar carbon sources to terpineol for growth. The study of *M. smegmatis* CYPs in bioremediation will be an important area of future research, as for the fungus *Phanerochaete chrysosporium* discussed later, for which 1% of the genes also encode CYPs. Common with the findings of many other genomic projects, the function of the mycobacterial CYPs remains unknown; however, as these are elucidated by gene knockouts, transcriptomics, proteomics, and metabolomics, there will be benefits for medical science and biotechnology.

## 5.2. Biodiversity in Streptomycetes

Streptomycetes are organisms with a complex life cycle, which involves the formation of a filamentous mycelium giving rise to aerial hyphae that produce spores. This, in part, explains the requirement for a larger genome in these bacteria that are also important producers of bioactive molecules (secondary metabolites). These metabolites represent about two thirds of the microbially derived compounds that include antibacterial (erythromycin, tetracycline), antifungal (amphotericin, nystatin), antiparasitic (avermectin), immunosuppressor (FK506), anti cancer (adriamycin), and herbicidal (bialaphos) compounds. Structural diversity is observed within all of the compounds and CYPs participate in oxidative tailoring of many of these, and thus play a key role in many of these pathways. The smell of wet earth on a spring day, resulting from geosmin, is also a product of actinomycetes/streptomycetes. Geosmin requires CYP for its biosynthesis and in *S. avermitilis* this is probably undertaken by CYP180A1[74]. With all these important biosynthetic pathways in which CYPs are known to participate (Table 13.4, Figure 13.6), there is of course interest in the cryptic pathways associated with the many orphan CYPs of streptomycete genomes for which function has yet to be detected. Apart from natural product biosynthesis, streptomycete CYPs have been identified as good biocatalysts with particular attention to xenobiotic metabolism by CYP105D1, identified

in *Streptomyces griseus*[77], and CYP105A1 from *S. griseolus*, which has been used to manipulate herbicide tolerance in plants[61]. Indeed, streptomycetes have been used as a source of drug metabolites by fermentation[80], and have also been used for stereo- and regio-specific biotransformations[81, 82].

Among the streptomycetes, two genomes have already been released into the public domain, for *S. coelicolor* A3(2) (ref. [72]) and for *S. avermitilis*[83]. The *S. coelicolor* genome of 7,825 open-reading frames contained 18 putative CYPs that were cataloged and expressed in a systematic study[73]. This laboratory strain is the model for streptomycetes and produces a number of secondary metabolites, although none are currently commercially important. The CYP roles are unclear, as the CYP51-like protein now called CYP170A1, is not involved in sterol biosynthesis and a gene deletion event was found not to be lethal[50]. Surprisingly, six of the eighteen CYPs are associated with operons with a conserved structure that have been called conservons[72]. In these, the first open-reading frame encodes a sensor kinase, then two open-reading frames of unknown function followed by a gene encoding an ATP-binding domain and finally, in some cases, encoding one or two *CYPs*. In conservon 10, the downstream *CYPs* are *CYP157A1* and *CYP154C1*; in conservon 11, the downstream *CYP* is *CYP157B1*; in conservon 12, the downstream *CYPs* are *CYP156A1* and *CYP154A1*; and in conservon 13, the downstream *CYP* is *CYP157C1*. The functions of these CYPs is intriguing, and as CYP154C1 can metabolize antibiotics, it could be related to a chemical defense and detoxification system[84]. Recently, a conservon from *S. griseus* was isolated containing a CYP157 homologue. Gene inactivation of the first gene of the conservon resulted in precocious formation of mycelium and secondary metabolism, suggesting this operon regulates the onset of differentiation[85].

**Table 13.4.** Streptomycete Cytochromes P450 Including CYP Assignments Where Available (www.drnelson.utmem.edu/P450.family.list.html).

| Streptomyces sp. | CYP identification | Bioactive molecule produced | Function |
|---|---|---|---|
| *S. griseolus* | CYP105A1, 105B1, 105C1 | | |
| *S. carbophilus* | CYP105A3 | | |
| *S. griseus* | CYP105D1, 105D2, 107F1 | | |
| *S. scerotialus* | CYP105D3 | | |
| *S. lividans* | CYP105D4 | | |
| *S. lavendulae* | CYP105F1, 107N1, **160A1** | Complestatin | Anti-HIV |
| *S. noursei* | CYP105H1, **161A1** | Nystatin | Antifungal |
| *S. tendae* | CYP105K1, **162A1** | Nikkomycin | Insecticidal |
| *S. fradiae* | CYP105L1, 113B1, **154B1** | Tylosin | Promotant |
| *S. clavuligerus* | CYP105M1 | | |
| *S. thermotolerans* | CYP107C1 | | |
| *S. erythraea* | **CYP107A1, 107B1** | Erythromycin | Antibacterial |
| *S. antibioticus* | **CYP107D1** | Oleandomycin | Antibacterial |
| *S. hygroscopius* | CYP107G1, **122A2, 122A3** | Rapamycin | Antibacterial |
| *S. venezuelae* | **PikC (PicK)** | Pikromycin | Antibacterial |
| *S. maritimus* | CYP107R1 | | |
| *S. peucetius* | CYP129A2, **131A1, 131A2** | Daunorubicin | Antitumor |
| *S. spheroides* | **CYP163A1** | Novobiocin | Antibacterial |
| *S. avermitilis* | **CYP171A1** | Avermectin | Antiparasitic |
| *S. acidscabies* | **TxtC** | Thaxtomin | Phytotoxin |
| *S. nodosus* | **Orf1, Orf2** | Amphotericin | Antifungal |

*Notes*: Many are involved in biosynthetic gene clusters of commercially important bioactive natural products and those implicated in this biosynthesis are in bold. The list does not include those found in the genomes of *Streptomyces coelicolor* and *Streptomyces avermitilis*.

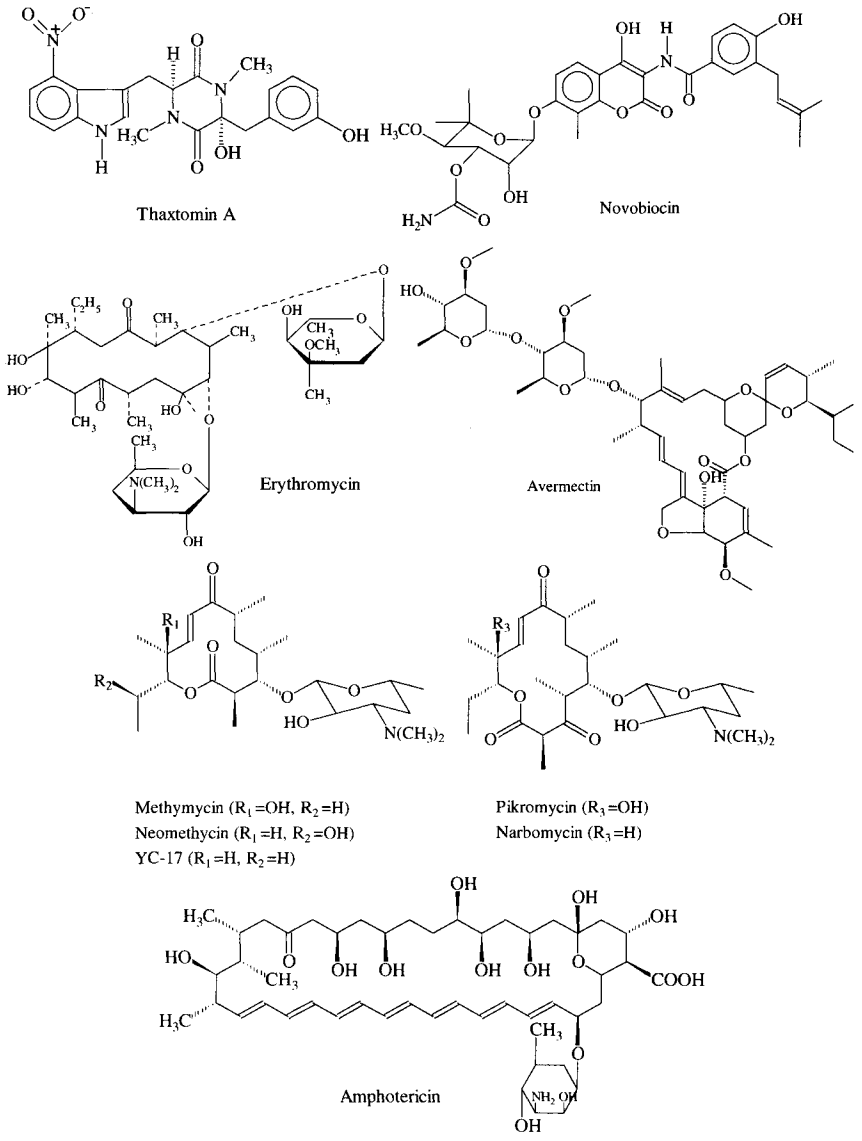



**Figure 13.6.** Some of the natural products used as drugs and produced by various streptomycetes.

Some of the functions of the *S. coelicolor* CYPs are in secondary metabolism operons, for example, *CYP158A2* located downstream of a Type III polyketide synthase and *CYP105N1* located downstream of a nonribosomal peptide synthase. These are currently subject to functional studies using the tools of gene disruption and metabolite/metabolome profiling, although not all the secondary metabolite genes identified have previously detected known end-products. The structural genomics of these CYPs is also in progress, with the first structure reported already for CYP154C1[84].

The second streptomycete genome completed has revealed 33 CYPs comprising 0.4% of genes. This industrial microorganism is important for the production of avermectin, an anti-helminthic agent. We have cataloged the *S. avermitilis*

CYPome and of note is the discovery of seven new families and two conservons containing CYPs[74]. The CYP157s within the conservons, as in *S. coelicolor*, deviate from the prior conservation in CYPs of an EXXR motif within the K-helix. CYP157A2 and CYP157C2 exhibited a $^{235}$EVLW and $^{257}$EQSLW motif, respectively. Together with the *S. coelicolor* CYP156A1 motif of $^{272}$STVR, the need for E and R is not essential, leaving only the heme cysteinyl ligand as the amino acid essential in all CYPs. These motifs are the subject of current experimental examination.

There were no clusters of *S. avermitilis* CYPs in a single subfamily except for *CYP105D6* and *CYP105D7*. These *CYP105D* forms seem associated with filipin biosynthesis, so that the roles of CYP105s in xenobiotic rather than secondary metabolism may well need reconsideration. CYP171A1 was involved in C8a oxidation in avermectin biosynthesis, CYP107W1 in oligomycin biosynthesis through oxidation at C12, and CYP180A1 is predicted to be involved in geosmin biosynthesis[74]. The closest homologue of CYP180A1 in *S. coelicolor* is CYP107U1, but geosmin is made by *S. coelicolor* and so must be synthesized using a CYP from a different family. When identified, it will require a modification of the nomenclature to place CYPs with common function in the same family, an illustration of some of the problems relating homology and CYP families to function.

Other CYPs identified in this genome include *CYP102s*, one of which encodes a fusion protein with a CYP and a reductase domain unlike the single *CYP102B1* found in *S. coelicolor A3(2)*. The *CYP102*s are also seen in bacillus species that are sporulating bacteria, but the endogenous function remains unclear. The *S. avermitilis* genome also contained a *CYP125*[74]. This had only previously been seen in mycobacteria, but was not present in *S. coelicolor* and was the first *CYP* family detected in two diverse actinomycete species.

Eleven of the *CYP*s from *S. avermitilis* were located in known gene clusters involved in secondary metabolism, including that of geosmin, avermectin, filipin, and pentalenolactone biosynthesis, and again, as in *S. coelicolor*, others appear to be involved in uncharacterized pathways of secondary metabolism. *CYP178A1* is in a cluster with a nonribosomal peptide synthase, *CYP107Y1* and *CYP181A1* are associated with a Type II polyketide synthase, *CYP154D1* is adjacent to an indole dioxygenase (and maybe involved in xenobiotic breakdown), while *CYP158A3* is adjacent to a Type III polyketide synthase, as is *CYP158A2* in *S. coelicolor*, which is likely to have a common function.

The emergence of further streptomycete genomes will add to our understanding of *CYP* evolution, and the numbers of *CYP*s in unknown pathways of secondary metabolism will reveal new natural products. Given the estimate that only 1% of microorganisms are culturable, the depth of the biocatalytic reservoir of CYPs becomes evident. The reason why the streptomycetes and mycobacteria have many CYPs is not clear, as other soil bacteria contain either small numbers of CYPs (Table 13.1) or none.

## 5.3. CYP biodiversity in Archaebacteria

CYPs also are found among the archaea in under a third of the genomes so far sequenced. Functional information about them is totally absent. Whether they arose later in evolution, after the appearance of oxygen, is also unclear. Their ability to maintain integrity in extreme conditions of temperature, and so on, could be useful in biotechnology as is the ability to function at low temperatures. The structures of two CYPs have been obtained. First, the CYP119 structure from *S. solfataricus* has been obtained and the heat stability of the protein has been ascribed to clusters of aromatic residues[69]. The second structure of CYP175A1 from the thermophilic *T. thermophilus* HB27 has also recently been solved[70].

# 6. Fungal CYPs

The yeast *Saccharomyces cerevisiae* has three *CYP* genes. *CYP51* was identified in 1987[35], *CYP57* was found in 1994 to be responsible for the synthesis of dityrosine, which is needed for the yeast spore wall[86], and *CYP61* was identified in 1995 as a sterol C22-desaturase in a proteomic study linking protein information to the emergence of this genome[87]. Fungal CYPs are generally class II associated with the endoplasmic reticulum and a single reductase drives all the CYPs, as in humans.

This limited diversity of CYPs was also seen in the fission yeast *Schizosaccharomyces pombe* that had *CYP51* and *CYP61* only, the minimal CYP requirement for ergosterol biosynthesis. The number of completed fungal genomes in the public domain is limited, but will expand in the coming years with sequences for major human and plant pathogens. This will rectify the current imbalance, given the economic, biomedical, biotechnological, and scientific reasons for obtaining fungal genome data and the large scientific community that will use this information. A shotgun analysis of the genome of *Candida albicans*, a major human pathogen, has been completed, but is not yet published at the time of writing. Our analysis of this genome reveals approximately 12 putative CYPs, including *CYP51* and *CYP61*, but also a novel orphan form *CYP501* and many members of the *CYP52* family. This was surprising, as these alkane utilization proteins are found in soil/environmental yeasts like *Candida tropicalis* and *Candida maltosa*. Perhaps these CYPs are involved in utilizing the host lipid in animals, or an otherwise unrecognized environmental niche for *C. albicans* exists. Another interesting observation is the presence of a *CYP56* homologue, that in *S. cerevisiae* is involved in dityrosine formation for the spore wall after yeast meiosis to produce tetrads[86]. However, dityrosine has been detected in the mitotic cell wall of *C. albicans*[88].

It was surprising that the *Neurospora crassa* genome contained 38 CYPs in a 40 MB genome. The genome of this filamentous fungus contained many CYPs from existing families, but as usual for a eukaryotic species of a previously unvisited biological type, it contained many new families of orphan function. The families observed were *CYP51*, *61A5*, *53A4*, *54*, *55A6*, *65B1*, *65C1*, *68D1*, *505A2*, and *507A1*, besides *CYP527A1* to *CYP553A1* which represent new families. No doubt similar numbers of CYPs will be identified in other fungi, but so far *P. chrysoporium* has many more for a fungal species (http://drnelson.utmem.edu/nelsonhomepage.html).

There is interest in using fungi in bioremediation and one of those that has been used commercially is the white-rot fungus *P. chrysosporium*. This is a basidiomycete, higher fungus with about 10,000 genes. Probing the unannotated genome revealed approximately 123 heme-binding motifs, so that approximately 1% of the genes of this microorganism encode for CYPs. This organism can degrade recalcitrant pollutants and the CYP system has been implicated in this activity[89–91]. The fungus is also commonly seen as a bracket fungus that can break down wood. As plants utilize only a small number (four) of CYPs to synthesize lignin it seems unlikely that all the CYPome of *P. chrysosporium* is involved in this aspect of metabolism, so much remains to be discovered about function of the orphan CYPs. Only one NADPH-reductase for the CYPs was present and this has been expressed and characterized[91, 92].

Fungal comparative genomics is in an early stage and, as with streptomycetes, much information about secondary metabolism is anticipated. Fifteen further fungi are to be sequenced, including the pathogen *Aspergillus fumigatus*. Also of interest will be the pathogenic basidiomycete *Cryptococcus neoformans* that, as with *P. chrysosporium*, may have a large CYPome as it is also associated with life in hollow eucalyptus trees and may therefore have evolved in a similar niche. Information on these projects is available on the web at the Sanger Center and TIGR sites.

Purification of fungal CYPs from cell extracts is a difficult task due to the usual low specific content, instability, and the presence of multiple forms. The fungal steroid hydroxylase CYPs have been studied and a polycyclic aromatic hydrocarbon hydroxylase[93, 94]. The emergence of genomes and the ability to express the CYPs present in *E. coli* or yeast has greatly facilitated their study, as will the application of transcription profiling.

In an early study by British Petroleum, the use of a *Candida* sp. producing single-cell protein from oil was envisaged. Although this became economically unviable during the 1970s with the rise in oil prices, it became apparent that CYP was responsible for the initial oxidation and that the CYPs responsible were from a new family, *CYP52*[95, 96]. Other *CYP52*s have been found and studied in many yeasts, including *C. maltosa* and *Yarrowia lipolytica*[97, 98]. Typically, many *CYP52*s are present in these yeast. Eight were found in *C. maltosa*, and knocking out four of these genes (also called *ALK* genes in the yeast nomenclature) prevented growth on n-alkane[97].

Other fungal CYP families identified include a benzoate *para*-hydroxylase from *Aspergillus niger* and a cycloheximide inducible *CYP54* from *N. crassa*[99, 100]. CYP55 ($P450_{nor}$) from *Fusarium oxysporum* represented a new class of CYP, as it is soluble, and carries out nitric oxide reduction without the need for a CYP-reductase (CPR) or a requirement for molecular oxygen. It was the first eukaryotic CYP to have its structure resolved and was a member of a new class of CYP[23].

CYP56 was found to be needed for dityrosine production for the spore walls of *S. cerevisiae*[86], while *CYP57* was identified among a group of six pea pathogenicity genes as a gene on a supernumary chromosome of *Nectria haematococca*. It plays a role in detoxifying the phytoalexin pisatin produced by the plant host[101]. CYP61, as mentioned above, is responsible for sterol C22-desaturation during ergosterol biosynthesis[87, 102–104]. Interestingly, some rice planthoppers and anobiid beetles use symbiotic yeast-like symbionts as a sterol source[105]. However, unlike in the beetles, the planthopper symbiont has a defective *CYP61* containing nonsense mutations and therefore accumulates ergosta-5,7,24(28)-trienol. The selection of this change is interesting in terms of the benefits in the relationship.

Some CYPs (CYP58, 59, 64) have been found to play a role in aflatoxin and mycotoxin biosynthesis[106–108], while *CYP68* is in a gene cluster involved in giberellin biosynthesis in *Giberella fujikuroi*[109], and another CYP is in the biosynthetic pathway of paxilline synthesis by *Penicillium paxilli*[110]. Fungal genome analysis will reveal many more *CYP*s involved in biosynthetic pathways of known and unknown natural products. Further novel CYP forms can also be anticipated, such as CYP505 from *F. oxysporum* that metabolizes fatty acids and is a membrane-bound CYP with a C-terminal CPR fusion[111].

# 7. Azole Antifungals and the Evolution of New Resistant Genes

Antifungal treatments have become increasingly important as fungal infections have become one of the top five most frequently encountered in the clinic. This increase is associated with the opportunistic nature of these fungal microorganisms that prey on the old and young, but also increasingly on patients in intensive care as well as with HIV, during cancer chemotherapy, and after organ transplantation[112]. The infections are by a variety of fungal species that also vary geographically, as well as demographically, but among the most important are *C. albicans*, increasingly other *Candida* spp. (such as *Candida glabrata* and *Candida krusei*), *C. neoformans, A. fumigatus, Histoplasma capsulatus, Pneumocystis carinii, Coccidiodes immitis, Penicillium italicum, Fusarium* (normally associated with diseases in plants), and even man's best friend, *S. cerevisiae*. Equally frequent are the skin and nail infections produced by dermatophytic fungi (*Trichophyton rubrum, Epidermophyton* spp., *Microsporum* spp.), which represent a significant market for drugs, albeit not because of life-threatening conditions. However, of the hundreds of thousands of fungal species, only about a hundred are reported as pathogens[113].

## 7.1. The Fungal CYP51 System

During the 1960s and 1970s a series of agrochemical fungicides and clinical antimycotics became available that were found, in studies with the plant pathogen *Ustilago maydis*, to be inhibiting sterol C14-demethylation[114]. This was also observed for azole compounds when treating *C. albicans* infections[115]. This step of sterol biosynthesis had been postulated to be a cytochrome P450-mediated activity[116, 117]. In pioneering work by Yoshida, Aoyama, and colleagues, CYP was purified and characterized from *S. cerevisiae* and, in a series of studies, they looked at the demethylation event occurring via three sequential monooxygenase reactions, and also at the enzymatic and electron transport requirements of the system that involved a typical eukaryotic NADPH-CPR[118, 119]. The reaction sequence was tested using recombinant *C. albicans* CYP51 protein and it was shown that the acyl-carbon bond cleavage occurred by a mechanism similar to those proposed by Akhtar and colleagues for the reactions involving CYP17 and CYP19[120, 121]. In other studies, CYP was purified from *S. cerevisiae* and shown to metabolize benzo(*a*)pyrene[122]. This was presumably the form that was responsible for activating pro-carcinogens in yeast genotoxicity

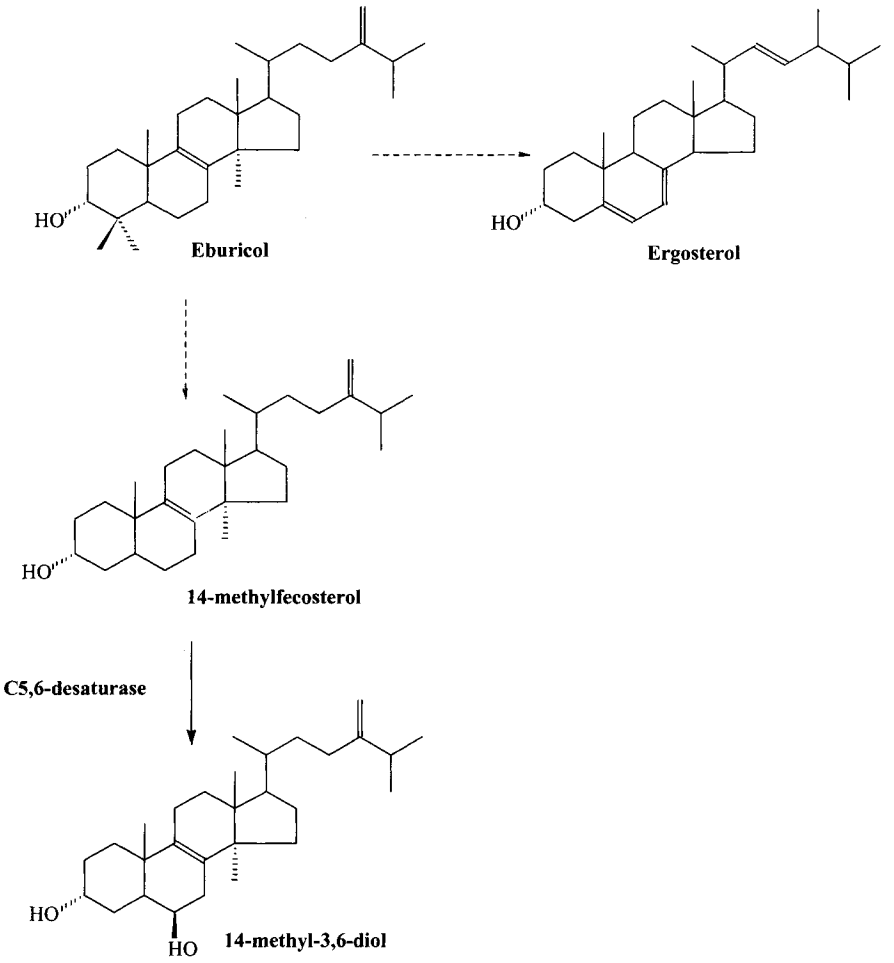



**Figure 13.7.** The sterols accumulating under azole treatment of *C. albicans* including the end-product, 14α-methylergosta-8,24(28)-dien-3β,6α-diol, that does not support growth and requires sterol C5-desaturase for biosynthesis. In most fungi, the substrate for CYP51 is more likely eburicol under normal conditions, and not lanosterol, although both are metabolized.

assays[123, 124]. The CYP purified was most likely CYP61, which has a low-level activity in benzo(*a*)pyrene metabolism as well as an endogenous activity in sterol C22-desaturation[49, 104, 125].

Genetic analysis of *CYP51* by gene disruption, the first for a CYP in any organism, demonstrated the essential nature of the gene product[35]. Chemical inhibition of CYP activity was also studied and genetic suppression of the effect of azole inhibition (by ketoconazole and fluconazole) was found to be mediated by sterol C5-desaturase mutants. These mutants changed the sterol accumulating under treatment from a fungistatic end-product, 14α-methylergosta-8,24(28)-dien-3β,6α-diol, to a functional end-product, 14α-methylfecosterol. This conversion required sterol C5-desaturase and therefore revealed sterol C5-desaturase to be implicated in the mode of action of azoles through introduction of a 6-OH group into 14-methyl sterol in a presumed attempted desaturation[126]. Subsequently, this was also found to suppress the genetic disruption of the *CYP51* locus[127, 128]. The discovery in the clinic of similar azole-resistant sterol

C5-desaturase mutants of *C. albicans* confirmed the central role of this biotransformation in the mode of action in a clinical setting[129, 130]. Sterol profiles of untreated and treated *C. albicans* illustrate the major sterols accumulating in wild-type and sterol C5-desaturase defective mutants (Figure 13.7).

It has also been found that the lethal effect of a sterol C4-methyl oxidase gene knockout can be suppressed by a mutation in *CYP51*[131]. These mutants accumulate lanosterol without further sterol metabolism and this indicates that lanosterol itself can support yeast growth despite previous sterol-feeding studies to the contrary[132]. Consequently, retention of a C14 methyl group on a sterol does not in itself make the sterol nonfunctional, as might be expected from the original events resulting in the evolution of the sterol pathway.

One additional point of interest here is the use of azole antifungals and CYP51 in proof of principal experiments within functional genomics, as pioneered using the yeast genome. Transcriptome studies with *S. cerevisiae* following treatment with fluconazole revealed increased CYP51 expression, showing the effect of the inhibitor and the usefulness of transcriptomics for determining drug mode of action[133, 134].

For activity, eukaryotic CYPs located in the endoplasmic reticulum require NADPH-CPR to provide the first and/or the second electron needed for the catalytic cycle (see Chapter 4)[135]. Gene disruption of yeast CPR was surprisingly not lethal[136], although the genome subsequently revealed no further *CPR* genes[137], and cells still synthesized ergosterol[138]. The source of electrons to support the requirement of CYP51and CYP61 for ergosterol biosynthesis has been studied and genetic evidence and reconstitution studies have shown that cytochrome b5 and cytochrome b5-reductase are responsible (Figure 13.7)[139]. This represents a difference from animals, where a *CPR* gene knockout in *Caenorhabditis elegans* and mouse is lethal[140,141]. Further differences exist in the yeast CPR, for which soluble forms have been produced that can support CYP activity, as shown in reconstitution assays and in genetic complementation studies using suitable yeast strains[138, 142]. Thus assumptions based on studies with mammalian systems may not be applicable across other Kingdoms or in other areas of CYP biology.

## 7.2. Azole Activity and Resistance in Fungi

In the early 1980s, it was first reported that resistance to the antifungal agent ketoconazole occurred in patients suffering chronic mucocutaneous candidiasis, but a general problem with antifungal resistance had never been encountered clinically. Resistance, had however, been seen in agriculture in the 1980s with the use of related fungicides generically termed demethylase inhibitors (DMIs).

Azoles bind to the heme of fungal CYP as a sixth ligand, as evidenced by the generation of Type II spectra using fungal microsomes, typically with a maximum at approximately 430 nm and a minimum at approximately 410 nm[143]. This interaction involves the N-3 of an imidazole ring or the N-4 of a triazole ring as a ligand to the heme, resulting in the formation of a low-spin, azole-bound complex. It had been known from 1972 that imidazoles could be CYP inhibitors[144]. A similar interaction was seen for the antifungal pyridyl compound buthiobate on binding to purified *S. cerevisiae* CYP51[145]. The studies on buthiobate showed saturation of the Type II spectra with a one-to-one ratio of the antifungal and CYP51, reflecting the high affinity of binding, although buthiobate and other azole antifungals can be displaced by carbon monoxide.

The orally administered antifungal drug ketoconazole was followed into clinical use by fluconazole and itraconazole, and more recently voriconazole, with further compounds still in clinical evaluation trials, including posaconazole and ravuconazole (Figure 13.8)[112]. In contrast, the range of agrochemicals that are in use is more diverse, and although newer compounds have been developed with alternate modes of action, resistance problems will emerge, necessitating a requirement for azoles. Resistance has emerged as a serious problem for agricultural and clinical use of azoles, but there is seemingly no potential causal link as with bacteria and concerns over the use of growth promotant antibiotics on the farm. The compounds have a selective effect on the pathogen over the effect on the human/plant CYP51, although direct comparisons at the level of the enzyme suggest that the sensitivities are closer to each other than might be anticipated (>10-fold)[146, 147]. Possibly CYP pools give a

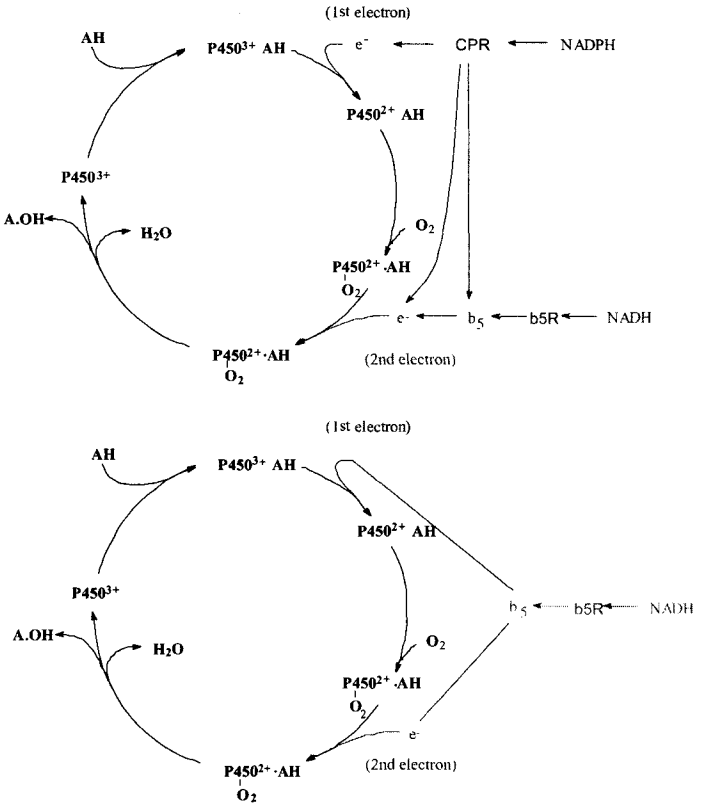



**Figure 13.8.** The typical CYP catalytic cycle with the potential for the second electron to be provided via cytochrome b5 rather than NADPH-cytochrome P450 reductase and the alternative observed in yeast where NADPH-cytochrome P450 reductase is not essential.

protective effect against CYP51 inhibitors in plants and humans.

With the emergence of the AIDS epidemic oropharengeal candidiasis became a serious problem and is often the presenting symptom indicating HIV infection, while in Southeast Asia it is often cryptococcosis. Aspergillosis is a serious health risk in organ-transplant patients and treatment success is much less than for common bacterial infections: here it appears that the latest azole drug voriconazole is superior to amphotericin, the previous drug of choice for this infection[112, 113]. With prolonged and often prophylactic use of azole drugs it is not surprising that resistance has emerged, requiring increasing drug treatment to control disease, especially for candidiasis. Molecular investigation of the mechanisms giving rise to resistance is ongoing the relevance of these for new azole drugs is being investigated. Some of these are shown in Figure 13.9.

Resistance to ketoconazole was studied in the 1980s in isolates obtained from patients suffering chronic mucocutaneous candidiasis. One of these, from the Darlington isolate(s), has been found to contain defective sterol C5-desaturase and altered CYP51 proteins, with the latter containing the substitutions Y132H and I471T[148]. Multiple mechanisms of resistance, between and within individual strains, is a common finding since the mid-1990s, when fluconazole resistance, primarily in HIV positive patients, became a clinical problem. First, some resistant strains of *C. albicans* from HIV patients were found to contain reduced concentrations of fluconazole[149, 150], and this was correlated with overexpression of transporters of the ABC superfamily, notably Cdr1p and Cdr2p

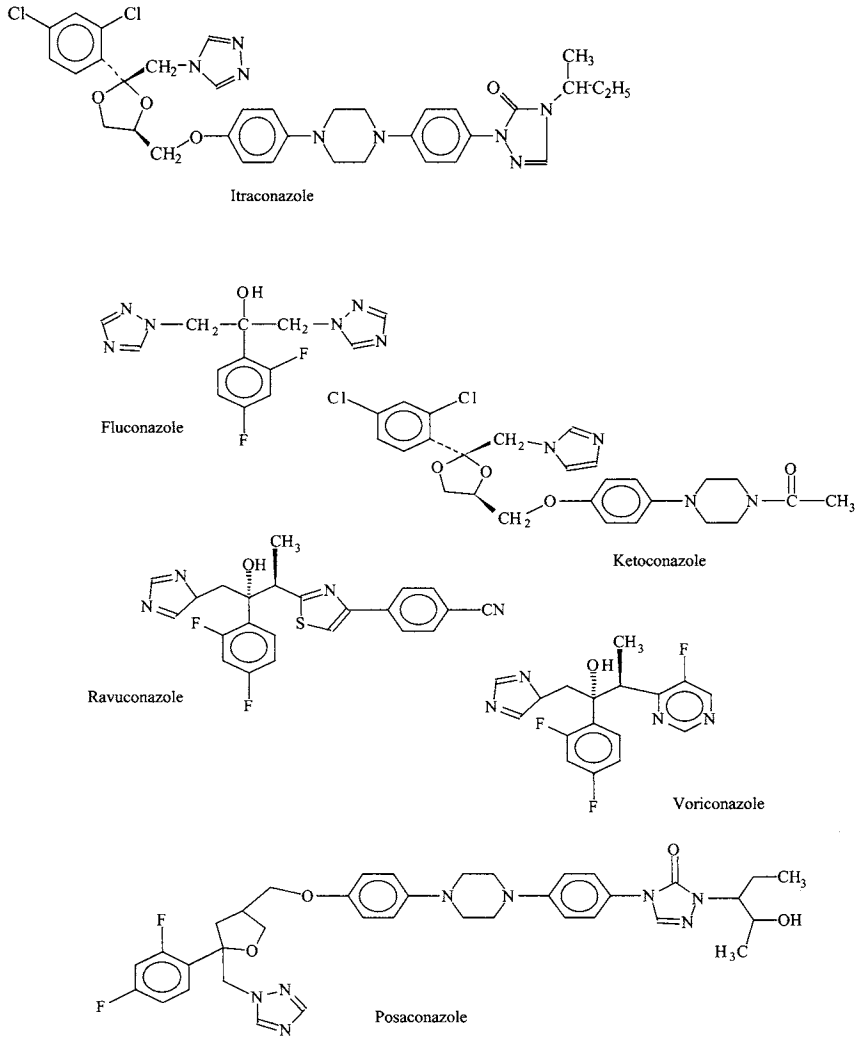



**Figure 13.9.** Some azole antifungals in current clinical use and under trial.

(Candida drug resistance proteins)[151, 152]. In addition, other studies implicated a major facilitator transporter of *C. albicans* in drug resistance (*MDR1*), as well as showing that *CDR1* and *CDR2* played a role in transporting the drug[151, 153, 154]. Other *C. albicans* resistant trains have shown defective sterol C5-desaturase activities, including some from AIDS patients and from patients suffering infections as a result of leukemia[129, 130].

Changes in CYP51 expression have been detected in resistant strains, but the role in causing resistance is unclear. For instance, in two studies on different series of isolates from two patients during the emergence of resistance, increased mRNA levels for CYP51 were observed in addition to increased transcription of drug transporters (CDR and MDR1) and substitutions in the amino acid sequence of CYP51 (R467K and G464S, respectively) [151, 154]. Overexpression of CYP51 on plasmids causes only a small increase in resistance, so this is not definitively identified as a cause of resistance[127]. In a different setting, a *Penicillium digitanum* resistant strain exhibited five repeats of a 126 bp sequence in the *CYP51*

promoter of a resistant strain, in comparison to one in a sensitive strain, and this may represent a resistance mechanism that results in enhanced *CYP51* expression[155]. *A. fumigatus* has two *CYP51* genes, as in other aspergilli[156], and this could also be the mechanism that makes it insensitive to fluconazole, although itraconazole resistance in *A. fumigatus* may be the result of mechanisms similar to those described in more detail here for *C. albicans*[157].

An isolate of *S. cerevisiae*, SG1, defective in sterol C14-demethylation, contained the substitution G310H, had an inactive protein, and was azole resistant[158]. The resistance was due to the second sterol C5-desaturase defect of the strain that suppressed the effect of the block in 14-demethylation. 14-Methyl-ergosta-24,(28)-dien-3,6-diol normally accumulates when 14-demethylation is blocked, but with a second defect in 5-desaturation, the functional sterol 14-methylfecosterol accumulates instead[127]. Other circumstantial evidence for mutant CYP51 resulting in azole resistance was observed[159], but it was not until 1997 that the first published evidence of changes in *C. albicans* CYP51 as a cause of resistance in practical settings appeared. These were reported for laboratory studies that had indicated that the change T315A in *C. albicans* CYP51 could alter fluconazole resistance when expressed in *S. cerevisiae*[160]. However, in a study of 18 resistant and 18 fluconazole resistant *C. albicans* isolates, a number of amino acid substitutions were observed in the resistant cohort suggestive of a causal link (Table 13.5). These included the change G464S observed in many subsequent investigations[161]. One particular problem was the absence of a parental isolate to compare resistant strains to, but a number of studies allowed sequential sets to be investigated that were obtained during the treatment of a patient and the emergence of resistance. In one set of 17 isolates, a number of molecular changes were observed, including increased transcription of Cdr1p ABC transporter that can confer cross-resistance to other azoles, and of Mdr1p, a major facilitator transporter, that confer fluconazole resistance[151]. During the study, increased transcript levels of CYP51 were also observed in later isolates. Furthermore, the substitution R467K in CYP51 produced an active protein with reduced affinity for fluconazole, as demonstrated by *in vitro* sterol biosynthesis in cell-free extracts and later in studies on the protein after expression in *S. cerevisiae*[162]. Another matched-set of *C. albicans* detected the appearance of G464S during the emergence of resistance[154]. As noted by White[153] and Loeffler *et al.*[161] the presence of homozygosity in this

**Table 13.5.** A List of Amino acid Substitutions Observed in CYP51 Among Clinical Fluconazole Resistant *Candida albicans*

| CYP51 substitution | References |
|---|---|
| F72L | Favre *et al.*, 1999 |
| F105L | Loeffler *et al.*, 1997 |
| D116E | Favre *et al.*, 1999 |
| F126L | Favre *et al.*, 1999 |
| K128T | Sanglard *et al.*, 1998 |
| G129A | Sanglard *et al.*, 1998 |
| Y132H | Sanglard *et al.*, 1998 |
| K143E | Favre *et al.*, 1999 |
| A149V | Marichal *et al.*, 1999 |
| D153E | Marichal *et al.*, 1999 |
| E165Y | Marichal *et al.*, 1999 |
| T229A | Favre *et al.*, 1999 |
| E266Q | Sanglard *et al.*, 1998 |
| E266D | Loeffler *et al.*, 1997 |
| | Favre *et al.*, 1999 |
| S279Y | Marichal *et al.*, 1999 |
| K287R | Loeffler *et al.*, 1997 |
| G307S | Perea *et al.*, 2001 |
| S405Y | Favre *et al.*, 1999 |
| S405F | Sanglard *et al.*, 1998 |
| | Favre *et al.*, 1999 |
| V437I | Sanglard *et al.*, 1998 |
| | Favre *et al.*, 1999 |
| G448E | Loeffler *et al.*, 1997 |
| F449L | Favre *et al.*, 1999 |
| G450E | Loeffler *et al.*, 1997 |
| V452A | Marichal *et al.*, 1999 |
| G464S | Loeffler *et al.*, 1997 |
| | Sanglard *et al.*, 1998 |
| | Marichal *et al.*, 1999 |
| G465S | Loeffler *et al.*, 1997 |
| | Marichal *et al.*, 1999 |
| R467K | White, 1997 |
| | Sanglard *et al.*, 1998 |
| I471T | Kakeya *et al.*, 2000 |
| V488I | Loeffler *et al.*, 1997 |

*Notes*: Not all these changes are known to cause resistance and only Y132H, G464S, and R467K have been subject to investigation at the level of protein.

diploid yeast implied two molecular events in the selection of resistant *CYP51*, an initial forward mutation followed by gene conversion of the second allele or (less likely on frequency grounds) a second identical mutational event in the second allele. This might be expected to produce higher level resistance than the heterozygous condition. Further point mutations in *CYP51* from fluconazole-resistant *C. albicans* were reported and yeast expression used to assess the resistance phenotype associated with the altered proteins and the altered sensitivity[152]. In many cases, more than one change from "wild-type" sequence existed and this allowed additive resistance changes and neutral *CYP51* polymorphisms to be detected. It is possible, as in reverse transcriptase substitutions in drug-resistant HIV, that selection could also involve changes that alter activity of the resistant protein and assist fitness.

Since these studies, a number of others have identified point mutations implicated in resistance using either *in vitro* sterol biosynthesis on cell-free extracts of the *C. albicans* strains, or more specifically, expression of the respective CYP51s and sensitivity testing using a heterologous host, *S. cerevisiae*. These studies have revealed a hugely diverse series of changes scattered across the protein and a list of these is included in Table 13.4[148, 154, 163–165]. Early molecular modeling of the CYP51 protein based on the CYP101 structure did not predict these residues as being important in azole interaction[166], although later models took into account a number of prokaryotic structures and existing information on fluconazole resistance mutations[167]. Interestingly, the latter model predicted a kink in the *C. albicans* CYP51 I-helix, and in the structure of *M. tuberculosis* CYP51 a break in the helix was found[44].

Of the mutations observed to-date, the helices B and B′, F126L, K128T, G129A, Y132H, K143R, F145L, and K147R would be close to the access channel. The Y132H mutation has been detected in a number of studies as well as in combination with different substitutions[148, 152, 163], and upon expression in yeast confers a 4-fold increase in fluconazole resistance and cross-resistance to itraconazole (2-fold) and ketoconazole (16-fold)[152]. E266D, R276H, D278E, and S279F are located at the end of the G-helix and G307S is the first substitution observed in the clinic to be related to resistance and located in the I-helix. Another cluster of residues associated with fluconazole resistance in *C. albicans* CYP51 are located before the heme-binding region consisting of G448E, F449L, G450E, and V452A, while G464S, G465S, R467K, and I471T are adjacent to the heme-cysteinyl ligand C470. While all these changes, together with further numerous unpublished alterations detected recently, need further characterization to define their effects on fluconazole binding, only F126, G464, and R467 are conserved across CYP51s. The numbers of mutants found with G464S and R467K were a surprise, as fluconazole binds above the heme as a sixth ligand with interaction of the N-1 substituent group within the active site. These substitutions below the plane of the heme are presumed to result in a change in the plane or orientation of the heme and to have a knockon effect on the location of the fluconazole bound above the heme[161, 162]. Surprisingly, in plant pathogens only a change in the equivalent residue to Y132 in *C. albicans* has so far been detected in grape powdery mildew among 19 resistant isolates[168]. This could make a simple diagnostic test for resistance feasible, as was also explored by Loeffler *et al.*[161] but the diversity of changes found in *C. albicans* CYP51 make this more complicated for clinical use.

To complete an understanding of the structural basis of resistance, biophysical studies will be required. A structure for a mammalian endoplasmic reticulum associated CYP has been achieved through expression of a soluble derivative and its crystallization[169, 170]. So far no reports of using *E. coli* and CYP51 for this purpose have occurred, but a soluble derivative that is active has been produced by expression of CYP51 containing an engineered protease site beyond the N-terminal membrane anchor. This may provide the route to resolving the structure if crystals can be obtained[142].

The nature of the diverse mutations giving rise to fluconazole resistance in *C. albicans* is remarkable, but mutations arose mainly in AIDS patients where resistance occurred in >10% during extended and prophylactic treatment[21]. With effective HIV treatment, this source of isolates has diminished as the immunocompetence of patients has improved. There will remain a problem, however, with continuation of azole use and development, and many facets of the resistance mechanisms remain to be unraveled, including

precise roles and effects of the diverse mutations in *CYP51*.

## 8. Conclusions

Microbial CYP biodiversity is presenting an unexpected challenge as the numbers of CYPs uncovered makes functional investigation through high-throughput genomics mandatory in addition to the more traditional, but essential approaches. The most facile organisms to study are the model organisms with well-established genetic systems, but many of the most interesting in terms of their CYP complements and environmental relevance, such as *P. chrysosporium,* will be important to tackle even if more recalcitrant. With many CYPs involved in metabolic pathways, an emphasis on changing CYPs to alter end-products will emerge. One of the most ambitious metabolic engineering projects to date involved the diversion of yeast ergosterol biosynthesis toward corticosteroid production, which involved deleting and adding CYPs to transgenic yeast[17]. Coupled to the ability to improve enzymes through processes of directed evolution, much will be possible in pathway improvement and invention as well as in biocatalysis[171].

Comparative genomics between close species and within strains will add to our information on the biological roles and evolution of CYPs. Contributions from currently unrepresented organisms from the fungi, protista, and algae will be of great interest in understanding CYP evolution. With 42 CYPs already detected in the slime-mould *Dictyostelium discoidium,* other protists are likely to have large numbers of CYPs. Where they are pathogenic, such as *Leischmeinia* and *Trypanosoma,* there will be the added interest of developing drugs to target these diseases via CYP inhibition, and azoles are already being used. The huge wave of CYP information from these projects will provide a major impetus to finding ways of establishing function and biotechnological uses in a systematic manner.

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## References

1. Klingenberg, M. (1958). Pigments of rat liver microsomes. *Arch. Biochem. Biophys.* **75**, 376–386.
2. Omura, T. and R. Sato (1962). A new cytochrome in liver microsomes. *J. Biol. Chem.* **237**, 1375–1376.
3. Estabrook, R.W., D.Y. Cooper, and O. Rosenthal (1963). The light reversible carbon monoxide inhibition of the steroid C21-hydroxylase system of the adrenal cortex. *Biochem. Z.* **338**, 741.
4. Lu, A.Y.H., K.W. Junk, and M.J. Coon (1969). Resolution of the cytochrome P450 in fatty acid ω-hydroxylation system into three components. *J. Biol. Chem.* **243**, 1331–1332.
5. Hanukoglu, I. (1992). Steroidogenic enzymes: Structure, function and regulation of expression. *J. Steroid Biochem. Mol. Biol.* **43**, 779–804.
6. Lindenmeyer, A. and L. Smith (1964). Cytochromes and other pigments of bakers yeast grown aerobically and anaerobically. *Biochem. Biophys. Acta.* **93**, 445–461.
7. Appleby, C.A. (1967). A soluble haemoprotein P450 from nitrogen-fixing rhizobium bacteroids. *Biochem. Biophys. Acta.* **147**, 399–402.
8. Katagiri, M., B.N. Ganguli, and I.C. Gunsalus (1968). A soluble cytochrome P450 functional in methylene hydroxylation. *J. Biol. Chem.* **243**, 3543–3546.
9. Tyson, C.A., J.D. Lipscomb, and I.C. Gunsalus (1972). The role of putidaredoxin and P450 cam in methylene hydroxylation. *J. Biol. Chem.* **247**, 5777–5784.
10. Rheinwald, J.G., A.M. Chakrabarty, and I.C. Gunsalus (1973). A transmissible plasmid controlling camphor oxidation in *Pseudomonas putida. Proc. Natl. Acad. Sci. USA* **70**, 885–889.
11. Sligar, S.G., P.G. Debrunner, J.D. Lipscomb, M.J. Namtvedt, and I.C. Gunsalus (1974). A role of the putidaredoxin COOH-terminus in P-450cam (cytochrome m) hydroxylations. *Proc. Natl. Acad. Sci. USA* **71**, 3906–3910.
12. Koga, H., B. Rauchfuss, and I.C. Gunsalus (1985). P450cam gene cloning and expression in *Pseudomonas putida* and *Escherichia coli. Biochem. Biophys. Res. Commun.* **130**, 412–417.
13. Narhi, L.O. and A.J. Fulco (1982). Phenobarbital induction of a soluble cytochrome P450 dependent fatty acid monooxygenase from *Bacillus megaterium. J. Biol. Chem.* **257**, 2147–2150.
14. Kelly, S.L., D.C. Lamb, M. Cannieux, D. Greetham, C.J. Jackson, T. Marczylo *et al.* (2001). An old

activity in the P450 superfamily (CYP51) and a new story of drugs and resistance. *Biochem. Soc. Trans.* **29**, 122–128.

15. Nelson, D.R., L. Koymans, T. Kamataki, J.J. Stegeman, R. Feyereisen, D.J. Waxman *et al.* (1996). P450 superfamily: Update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* **6**, 1–42.
16. Kelly, S.L., D.C. Lamb, and D.E. Kelly (1999). Inhibitors of CYP51 as antifungal agents and resistance to azole antifungals. In (E. Arinc, J.B. Schenkman, and E. Hodgson (eds), *Molecular and Applied Aspects of Oxidative Drug Metabolising Enzymes*. Plenum, New York, pp. 157–172.
17. Szczebara, F.M., C. Chandelier, C. Villeret, A. Masurel, S. Bourot, C. Duport *et al.* (2003). Total biosynthesis of hydrocortisone from a simple carbon source. *Nat. Biotechnol.* **21**, 143–149.
18. Rodriguez-Saiz, M., J.L. Barredo, M.A. Moreno, J.M. Fernandez-Canon, M.A. Penalva, and B. Diez (2001). Reduced function of a phenylacetate-oxidising cytochrome P450 caused by strong genetic improvement in early phylogeny of penicillin-producing strains. *J. Bact.* **183**, 5465–5471.
19. Mingot, J.M., M.A. Penalva, and J.M. Fernandez-Canon (1999). Disruption of phacA, an *Aspergillus nidulans* gene encoding a novel cytochrome P450 monooxygenase catalysing phenylacetate 2-hydroxylation results in penicillin overproduction. *J. Biol. Chem.* **274**, 14545–14550.
20. Smith, K.E., F. Ahmed, and T. Antoniou (1993). Microbial transformation of steroids. *Biochem. Soc. Trans.* **21**, 1077–1080.
21. Lupetti, A., R. Danesi, M. Campa, M. Del Tacca, and S. Kelly (2002). Molecular basis of resistance to azole antifungals. *Trends Mol. Med.* **8**, 76–81.
22. Wen, L.P. and A.J. Fulco (1987). Cloning of the gene encoding a catalytically self-sufficient cytochrome P450 fatty-acid monooxygenase induced by barbiturates in *Bacillus megaterium* and its functional expression and regulation in heterologous (*Escherichia coli*) and homologous (*Bacillus megaterium*) hosts. *J. Biol. Chem.* **262**, 6676–6682.
23. Park, S.Y., H. Shimizu, S. Adachi, A. Nakagawa, I. Tanaka, K. Nakahara *et al.* (1997). Crystal structure of nitric oxide reductase from denitrifying fungus *Fusarium oxysporum. Nat. Struct. Biol.* **4**, 827–832.
24. Kelly, S.L., D.C. Lamb, C.J. Jackson, A.G.S. Warrilow, and D.E. Kelly (2003). The biodiversity of microbial cytochromes P450. *Adv. Microb. Physiol.* **47**, 131–186.
25. Hawkes, D.B., G.W. Adams, A.L. Burlingame, P.R.O. de Montellano, and J.J. De Voss (2002). Cytochrome P450(cin) (CYP176A1), isolation, expression and characterization. *J. Biol. Chem.* **277**, 27725–27732.
26. Roberts, G.A., G. Grogan, A. Greter, S.L. Flitsch, and N.J. Turner (2002). Identification of a new class of cytochrome P450 from a *Rhodococcus* sp. *J. Bact.* **184**, 3898–3908.
27. Jackson, C.J., D.C. Lamb, T. Marczylo, A.G.S. Warrilow, N.J. Manning, D.J. Lowe *et al.* (2002). A novel sterol 14α-demethylase/ferredoxin fusion protein (MCCYP51FX) from *Methylococcus capsulatus* represents a new class of the cytochrome P450 superfamily. *J. Biol. Chem.* **277**, 46959–46965.
28. Seth-Smith, H.M.B., S.J. Rosser, A. Basran, E.R. Travis, E.R. Dabbs, S. Nicklin *et al.* (2002). Cloning, sequencing and characterisation of the hexahydro-1,3,5-trinitro-1,3,5-triazine degradation gene cluster from *Rhodococcus rhodochrous. Appl. Env. Microbiol.* **68**, 4764–4771.
29. De Mot, R. and A.H. Parret (2002). A novel class of self-sufficient cytochrome P450 monooxygenase in prokaryotes. *Trends Microbiol.* **10**, 502–508.
30. Gonzalez, F.J. and D.W. Nebert (1990). Evolution of the P450 gene superfamily: Animal–plant warfare, molecular drive and human genetic differences in drug oxidation. *Trends Genet.* **6**, 182–186.
31. Nelson, D.R. (1999). Cytochrome P450 and the individuality of species. *Arch. Biochem. Biophys.* **369**, 1–10.
32. Yoshida, Y, Y. Aoyama, M. Noshiro, and O. Gotoh (2000). Sterol 14-demethylase P450 (CYP51) provides a breakthrough for the discussion of the evolution of cytochrome P450 gene superfamily. *Biochem. Biophys. Res. Commun.* **273**, 799–804.
33. Jackson, C.J., D.C. Lamb, T.H. Marczylo, J.E. Parker, N.J. Manning, D.E. Kelly *et al.* (2003). Conservation and cloning of CYP51, a sterol 14α-demethylase from *Mycobacterium smegmatis. Biochem. Biophys. Res. Commun.* **307**, 558–563.
34. Stoke, J.E. and J.J. De Voss (2000). Expression, purification and characterisation of biol: A carbon–carbon bond cleaving cytochrome P450 involved in biotin biosynthesis in *Bacillus subtilis. Arch. Biochem. Biophys.* **384**, 351–360.
35. Kalb, V.F., C.W. Woods, T.G. Turi, C.R. Dey, T.R. Sutter, and J.C. Loper, (1987). Primary structure of P450 lanosterol demethylase gene from *Sacchaaromyces cerevisiae. DNA* **6**, 529–537.
36. Aoyama, Y., Y. Funae, M. Noshiro, T. Horiuchi, and Y. Yoshida (1994). Occurence of a P450 showing high homology to yeast lanosterol 14-demethylase (P45014DM) in the rat liver. *Biochem. Biophys. Res. Commun.* **201**, 1320–1326.
37. Stromstedt, M., D. Rozman, and M.R. Waterman (1996). The ubiquitously expressed human CYP51 encodes lanosterol 14-demethylase, a cytochrome P450 whose expression is regulated by oxysterols. *Arch. Biochem. Biophys.* **329**, 73–81.

38. Bak, S., R.A. Kahn, C.E. Olsen, and B. Halkier (1997). Cloning and expression in *E. coli* of the obtusifoliol of *Sorghum bicolor* (L.) Moench, a cytochrome P450 orthologous to the sterol 14α-demethylases (CYP51) from mammals and fungi. *Plant J.* **11**, 191–201.
39. Lamb, D.C., D.E. Kelly, and S.L. Kelly (1998). Molecular diversity of sterol 14α-demethylase substrates in plants, fungi and humans. *FEBS Lett.* **425**, 263–265.
40. Wickramashighe, R.H. and C.A. Villee (1975). Early role during chemical evolution for cytochrome P450 in oxygen detoxification. *Nature* **256**, 509–510.
41. Godzina, S.M., M.A. Lovato, M.M. Meyer, K.A. Foster, W.K. Wilson, W. Gu *et al.* (2000). Cloning and characterisation of the *Dictyostelium discoideum*. *Lipids* **35**, 249–255.
42. Joubert, B.M., F.S. Buckner, and S.P.T. Matsuda (2001). Trypanosome and animal lanosterol synthase use different catalytic motifs. *Org. Lett.* **3**, 1957–1960.
43. Bellamine, A., A.T. Mangla, W.D. Nes, and M.R. Waterman (1999). Characterisation and catalytic properties of the sterol 14α-demethylase from *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. USA* **96**, 8937–8942.
44. Podust, L.M., T.L. Poulos, and M.R. Waterman (2001). Crystal structure cytochrome P450 14α-sterol demethylase from *Mycobacterium tuberculosis* in complex with azole inhibitors. *Proc. Natl. Acad. Sci. USA* **98**, 3068–3073.
45. Bird, C.W., J.M. Lynch, F.J. Pirt, W.W. Reid, C.J.W Brooks, and B.S. Middleditch (1971). Steroids and squalene in *Methylococcus capsulatus* grown on methane. *Nature* **230**, 473–474.
46. Bode, H.B., B. Zeggel, B. Silakowski, S.C. Wenzel, H. Reichenbach, and R. Muller (2003). Steroid biosynthesis in prokaryotes: Identification of myxobacterial steroids and cloning of the first bacterial 2,3(S)-oxidosqualene cyclase from the myxobacterium *Stigmatella aurantiaca*. *Mol. Microbiol.* **47**, 471–481.
47. Buckner, F.S., A.J. Wilson, T.C. White, and W.C. Van Voorhis (1998). Induction of resistance to azole drugs in *Trypanosoma cruzi*. *Antimicrob. Agents Chemother.* **42**, 3245–3250.
48. Salimova, E., A. Boschetti, W. Eichenberger, and L. Lutova (1999). Sterol mutants of *Chlamydomonas reinhardtii*: Characterisation of three strains deficient in C24(28) reductase. *Plant Physiol. Biochem.* **37**, 241–249.
49. Kelly, S.L., D.C. Lamb, and D.E. Kelly (1997). Sterol 22-desaturase, cytochrome P450 61, possesses activity in xenobiotic metabolism *FEBS Lett.* **412**, 233–235.
50. Lamb, D.C., K. Fowler, T. Kieser, N.J. Manning, L.M. Podust, M.R. Waterman *et al.* (2002). Sterol 14α-demethylase activity in *Streptomyces coelicolor* A3(2) is associated with an unusual member of the CYP51 gene family. *Biochem. J.* **364**, 555–562.
51. Jackson, C.J., D.C. Lamb, D.E. Kelly, and S.L. Kelly (2000). Bactericidal and inhibitory effects of azole antifungal compounds on *Mycobacterium smegmatis*. *FEMS Lett.* **192**, 159–162.
52. Guardiola-Diaz, H.M., L.A. Foster, D. Mushrush, and A.D.N. Vaz (2001). Azole-binding to a novel cytochrome P450 from *Mycobacterium tuberculosis*: Implications for treatment of tuberculosis. *Biochem. Pharmacol.* **61**, 1463–1470.
53. McLean, K.J., M.R. Cheeseman, S.L. Rivers, A. Richmond, D. Leys, S.K. Chapman *et al.* (2002). Expression, purification and spectroscopic characterisation of the cytochrome P450 CYP121 from *Mycobacterium tuberculosis*. *J. Inorg. Biochem.* **91**, 527–541.
54. Cole, S.T., K. Eiglmeier, J. Parkhill, K.D. James, N.R. Thomson, P.R. Wheeler *et al.* (2001). Massive gene decay in leprosy bacillus. *Nature* **409**, 1007–1011.
55. Tang, L., S. Shah, L. Chung, J. Carney, L. Katz, C. Khosla *et al.* (2000). Cloning and expression of the epothilone gene cluster. *Science* **287**, 640–642.
56. August, P.R., L. Tang, Y.J. Yoon, S. Ning, R. Muller, T-W. Yu *et al.* (1998). Biosynthesis of the ansamycin antibiotic rifamycin: Deductions from the molecular analysis of the rif biosynthetic cluster of *Amycolatopsis mediteranei* S699. *Chem. Biol.* **5**, 69–79.
57. Poulos, T.L., B.C. Finzel, I.C. Gunsalus, G.C. Wagner, and J. Kraut (1985). The 2.6A crystal structure of *Pseudomonas putida* cytochrome P450. *J. Biol. Chem.* **260**, 16122–16130.
58. Poulos, T.L., B.C. Finzel, and A.J. Howard (1987). High-resolution crystal structure of cytochrome P450cam. *J. Mol. Biol.* **195**, 687–700.
59. Peterson, J.A., J.Y. Lu, J. Geisselsoder, S. Graham Lorence, C. Carmona, F. Witney *et al.* (1992). Cytochrome P450 terp-isolation and purification of the protein and cloning and sequencing of its operon. *J. Biol. Chem.* **267**, 14193–14203.
60. Nagy, I., F. Compernolle, K. Ghys, J. VanderLeyden, and R. Demot (1995). A single cytochrome P450 system is involved in degradation of the herbicides EPTC (S-ethyl dipropylthiocarbamate) and atrazine by *Rhodococcus* sp. strain NI86/21. *J. Bact.* **61**, 2056–2060.
61. O'Keefe, D.P., J.M. Tepperman, C. Dean, K.J. Leto, D.L. Erbes, and J.T. O'Dell (1994). Plant expression of a bacterial cytochrome P450 that catalyzes activation of a sulfonylurea pro-herbicide. *Plant Physiol.* **105**, 473–482.

62. Ruettinger, R.T. and A.J. Fulco (1981). Epoxidation of unsaturated fatty acids by a soluble cytochrome P-450-dependent system from *Bacillus megaterium*. *J. Biol. Chem.* **256**, 5728–5734.
63. Narhi, L.O. and A.J. Fulco (1986). Characterization of a catalytically self-sufficient 119,000-dalton cytochrome P-450 monooxygenase induced by barbiturates in *Bacillus megaterium*. *J. Biol. Chem.* **261**, 7160–7169.
64. Narhi, L.O. and A.J. Fulco (1987). Identification and characterization of two functional domains in cytochrome P-450BM-3, a catalytically self-sufficient monooxygenase induced by barbiturates in *Bacillus megaterium*. *J. Biol. Chem.* **262**, 6683–6690.
65. Ravichandran, K.G., S.S. Boddupalli, C.A. Hasermann, J.A. Peterson, and J. Deisenhofer (1993). Crystal structure of hemoprotein domain of P450BM-3, a prototype for microsomal P450's. *Science* **261**, 731–736.
66. Munro, A.W. (1994). The role of Tryptophan 97 of cytochrome P450 BM3 from *Bacillus megaterium* in catalytic function. *Biochem. J.* **303**, 423–428.
67. Yeom, H., S.G. Sligar, H.Y. Li, T.L. Poulos, and A.J. Fulco (1995). The role of Thr268 in oxygen activation in cytochrome P450 BM3. *Biochemistry* **34**, 14733–14740.
68. Klein, M.L. and A.J. Fulco (1993). Critical residues involved in FMN binding and catalytic activity in cytochrome P450BM3. *J. Biol. Chem.* **268**, 7553–7561.
69. Yano, J.K., L.S. Koo, D.J. Schuller, H. Li, P.R. Ortiz de Montellano, and T.L. Poulos (2000). Crystal structure of a thermophilic cytochrome P450 from the archaeon *Sulfolobus solfataricus*. *J. Biol. Chem.* **275**, 31086–31092.
70. Yano, J.K., F. Blasco, H. Li, R.D. Schmid, A. Henne, and T.L. Poulos (2003). Preliminary characterization and crystal structure of a thermostable cytochrome P450 from *Thermus thermophilus*. *J. Biol. Chem.* **278**, 608–616.
71. Cole, S.T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris *et al.* (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**, 537–554.
72. Bentley, S.D., K.F. Chater, A.M. Cerdeno-Tarraga, G.L. Challis, N.R. Thomson, K.D. James *et al.* (2002). Complete sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* **417**, 141–147.
73. Lamb, D.C., T. Skaug, H.-L. Song, C.J. Jackson, L. Podust, M.R. Waterman *et al.* (2002). The cytochrome P450 complement (CYPome) of *Streptomyces coelicolor* A3(2). *J. Biol. Chem.* **277**, 24000–24005.
74. Lamb, D.C., H. Ikeda, D.R. Nelson, J. Ishikawa, T. Skaug, Jackson C.J. *et al.* (2003). Cytochrome P450 complement of the avermectin-producer *Strepto-myces avermitilis* and comparison to that of *Streptomyces coelicolor* A3(2). *Biochem. Biophys. Res. Commun.* **307**, 610–619.
75. Sariaslani, F.S. and J.P. Rosazza (1983). Novel biotransformations of 7-ethoxycoumarin by *Streptomyces griseus*. *Appl. Environ. Microbiol.* **46**, 468–474.
76. Sariaslani, F.S. and J.P. Rosazza (1983). Microbial transformation of natural anti-tumor agents—products of rotenone and dihydrorotenone transformation. *Appl. Environ. Microbiol.* **49**, 451–452.
77. Trower, M.K., R. Lenstra, C. Omer, S.E. Buchholz, and F.S. Sariaslani (1992). Cloning, nucleotide sequence determination and expression of the genes encoding cytochrome P450soy (SOYC) and ferredoxinSOY (SOYC) from *Steptomyces griseus*. *Mol. Microbiol.* **6**, 2125–2134.
78. O'Keefe, D.P., S.M.C. Lau, and P.A. Harder (1993). Efficiency and substrate-specificity of *Streptomyces griseolus* cytochrome P450SU1 and cytochrome P450SU2 monooxygenase reactions. *Biochem. Soc. Trans.* **21**, 1073–1077.
79. Poupin, P., V. Ducrocq, S. Hallier-Soulier, and N. Truffeut (1999). Cloning and characterisation of the genes encoding a cytochrome P450 (PipA) involved in piperidine and pyrrolidine utilisation and its regulatory protein (PipR) in *Mycobacterium smegmatis* mc(2)155. *J. Bacteriol.* **181**, 3419–3426.
80. Cannell, R.J.P., A.R. Knaggs, M.J. Dawson, G.R. Manchee, P.J. Eddershaw, I. Waterhouse *et al.* (1995). Microbial biotransformation of the angiotensin II antagonist GR117289 by *Streptomyces rimosus* to identify a mammalian metabolite. *Drug Metab. Disp.* **23**, 724–729.
81. Berrie, J.R., R.A.D. Williams, and K.E. Smith (1999). Microbial transformations of steroids-XI. Progesterone transformation by *Streptomyces roseo* chromogenes-purification and characterisation of the 16 alpha-hydroxylase system. *J. Steroid Biochem.* **71**, 153–165.
82. Ito, S., T. Hata, I. Watanabe, T. Matsuoka, and N. Serizawa (1997). Crystal structure of cytochrome P450sca from *Streptomyces carbophilus* involved in production of pravastatin, a cholesterol lowering drug. *FASEB J.* **11**, 36.
83. Ikeda, H., J. Ishikawa, A. Hanamoto, M. Shinose, H. Kikuchi, T. Shiba *et al.* (2003). Complete genome sequence and comparative analysis of the industrial microorganisms *Streptomyces avermitilis Organism. Nat. Biotechnol.* **21**, 526–531.
84. Podust, L.M., Y. Kim, M. Arase, B.A. Neely, B.J. Beck, H. Bach *et al.* (2003). The 1.92-angstrom structure of *Streptomyces coelicolor* A3(2) CYP154C1—a new monooxygenase that

functionalises macrolide ring systems. *J. Biol. Chem.* **278**, 12214–12221.
85. Komatsu, M., Y. Kuwara, A. Hiroishi, K. Hosono, T. Beppu, and K. Ueda (2003). Cloning of the conserved regulatory operon by its aerial mycelium-inducing activity in an amfR mutant of *Streptomyces griseus*. *Gene* **306**, 79–89.
86. Briza, P., M. Eckerstorfer, and M. Breitenbach (1994). The sporulation-specific enzymes encoded by the DIT1 and DIT2 genes catalyze a two-step reaction leading to a soluble LL-dityrosine-containing precursor of the yeast spore wall. *Proc. Natl. Acad. Sci. USA* **91**, 4524–4528.
87. Kelly, S.L., D.C. Lamb, A.J. Corran, B.C. Baldwin, L.W. Parks, and D.E. Kelly (1995). Purification and reconstitution of activity of *Saccharomyces cerevisiae* P450 61, a sterol 22-desaturase. *FEBS Lett.* **377**, 217–220.
88. Smail, E.H., P. Briza, A. Panagos, and L. Berenfeld (1995). *Candida albicans* cell walls contain the fluorescent cross-linking amino acid dityrosine. *Inf. Immun.* **63**, 4078–4083.
89. Masaphy, S., D. Levanon, Y. Henis, K. Venkateswarlu, and S.L. Kelly (1996). Evidence for cytochrome P450 and P450-mediated benzo(a)pyrene hydroxylation in the white-rot fungus *Phanerochaete chrysosporium*. *FEMS Lett.* **135**, 51–55.
90. Kullman, S.W. and F. Matsumura (1997). Identification of a novel cytochrome P-450 gene from the white-rot fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **63**, 2741–2746.
91. Yadav, J.S. and J.C. Loper (2000). Cytochrome P450 oxidoreductase gene and its differentially terminated cDNAs from the white-rot fungus *Phanerochaete chrysosporium*. *Curr. Genet.* **37**, 65–73.
92. Warrilow, A.G.S., D.C. Lamb, D.E. Kelly, and S.L. Kelly (2002). *Phanerochaete chrysosporium* NADPH-cytochrome P450 reductase kinetic mechanism. *Biochem. Biophys. Res. Commun.* **299**, 189–195.
93. Breskvar, K., B. Cresnar, and T. Hudnik-Plevnik (1987). Resolution and reconstitution of cytochrome P450 containing steroid hydroxylation system of *Rhizopus nigricans*. *J. Steroid Biochem.* **14**, 395–399.
94. Masaphy, S., D.C. Lamb, and S.L. Kelly (1999). Purification and characterization of a benzo(a)pyrene hydroxylase from *Pleurotus pulmonarius*. *Biochem. Biophys. Res. Commun.* **266**, 326–329.
95. Lebeault, J.M., E.T. Lode, and M.J. Coon (1971). Fatty acid and hydrocarbon hydroxylation in yeast: Role of cytochrome P450 in *Candida tropicalis*. *Biochem. Biophys. Res. Commun.* **42**, 413–419.
96. Sanglard, D. and J.C. Loper (1989). Characterisation of the alkane-inducible cytochrome P450 (P450ALK) gene from the yeast *Candida tropicalis*—identification of a new P450-gene family, Gene **76**, 121–136.
97. Ohkuma, M., T. Zimmer, T. Iida, W.H. Schunck, A. Ohta, and M. Takagi (1998). Isozyme function of n-alkane-inducible cytochromes P450 in *Candida maltosa* revealed by sequential gene disruption. *J. Biol. Chem.* **273**, 3948–3953.
98. Iida, T., A. Ohta, and M. Takagi (1998). Cloning and characterisation of an n-alkane inducible cytochrome P450 gene essential for n-decane assimilation by *Yarrowia lipolytica,*. *Yeast* **14**, 1387–1397.
99. VanGorcom, R.F., J.G. Boschloo, A. Kuijvenhoven, J. Lange, A.J. vanVark, C.J. Bos *et al.* (1990). Isolation and molecular characterisation of the benzoate para-hydroxylase gene (bphA) of *Aspergillus niger*: A member of a new gene family of the cytochrome P450 superfamily. *Mol. Gen. Genet.* **223**, 192–197.
100. Attar, R.M., E. Grotewold, G.E. Taccioli, G.O. Aisemberg, H.N. Torres, and N.D. Judewicz (1989). A cycloheximide-inducible gene of *Neurospora crassa* belongs to the cytochrome P450 superfamily. *Nucleic Acids Res.* **17**, 7535–7536.
101. Han, Y.N., X.G. Liu, U. Benny, H.C. Kistler, and H.D. VanEtten (2001). Genes determining pathogenicity to pea are clustered on a supernumerary chromosome in the fungal plant pathogen *Nectria haematococca*. *Plant J.* **25**, 305–314.
102. Hata, S., T. Nishino, M. Komori, and H. Katsuki (1981). Involvement of cytochrome P-450 in delta 22-desaturation in ergosterol biosynthesis of yeast. *Biochem. Biophys. Res. Commun.* **103**, 272–277.
103. Skaggs, B.A., J.F. Alexander, C.A. Pierson, K.S. Schweitzer, K.T. Chun, C. Koegel *et al.* (1996). Cloning and characterization of the *Saccharomyces cerevisiae* C-22 sterol desaturase gene, encoding a second cytochrome P-450 involved in ergosterol biosynthesis. *Gene* **169**, 105–109.
104. Kelly, S.L., D.C. Lamb, B.C. Baldwin, A.J. Corran, and D.E. Kelly (1997). Characterisation of *Saccharomyces cerevisiae* CYP61, a sterol delta 22-desaturase, and inhibition by azole antifungal agents. *J. Biol. Chem.* **272**, 9986–9988.
105. Noda, H. and Y. Koizumi (2003). Sterol biosynthesis by symbiotes: Cytochrome P450 sterol C5-desaturase genes from yeastlike symbiotes of rice planthoppers and anobiid beetles. *Insect Biochem. Mol. Biol.* **33**, 649–658.
106. Hohn, T.M., A.E. Desjardins, and S.P. McCormick (1995). The TRI4 gene of *Fusarium sporotrichoides* encodes a cytochrome P450 monooxygenase involved in trichothene biosynthesis. *Mol. Gen. Genet.* **248**, 95–102.

107. Yu, J.J., P.K. Chang, K.C. Ehrlich, B. Montalbano, J.M. Dyer, D. Bhatnagar *et al.* (1998). Characterisation of the critical amino acids of an *Aspergillus parasiticus* cytochrome P450 monooxygenase encoded by ordA that is involved in the biosynthesis of aflatoxins B-1, G(1), B-2 and G(2). *Appl. Env. Microbiol.* **64**, 4834–4841.
108. Yu, J., P.K. Chang, D. Bhatnagar, and T.E. Cleveland (2000). Genes encoding cytochrome P450 and monooxygenase enzymes define one end of the aflatoxin pathway gene cluster in *Aspergillus parasiticus*. *Appl. Micro. Biotech.* **53**, 583–590.
109. Tudzynski, B., P. Hedden, E. Carrera, and P. Gaskin (2001). The P450-4 gene of *Gibberella fujikuroi* encodes ent-kaurene oxidase in the gibberellin biosynthesis pathway *App. Environ. Microbiol.* **67**, 3514–3522.
110. Young, C., L. McMillan, E. Telfer, and B. Scott (2001). Molecular cloning and genetic analysis of an indole-diterpene gene cluster from *Penicillium paxilli*. *Mol. Microbiol.* **39**, 754–764.
111. Kitazume, T., N. Takaya, N. Nakayama, and H. Shoun (2000). *Fusarium oxysporum* fatty acid subterminal hydroxylase (CYP505) is a membrane-bound counterpart of *Bacillus megaterium* P450BM3. *J. Biol. Chem.* **275**, 39734–39740.
112. Lamb, D.C., D.E. Kelly, and S.L. Kelly (1999). Molecular aspects of azole antifungal action and resistance. *Drug Resist. Updates* **2**, 90–402.
113. Odds, F.C. (1996). Antifungal therapy. In C.C. Kibbler, D.W.R. Mackenzie, and F.C. Odds (eds), *Principles and Practise of Clinical Mycology*, John Wiley & Sons, Chichester. pp. 1–6.
114. Ragsdale, N.N. and H.D. Sisler (1972). Mode of action of triarimol in *Ustilago maydis*. *Biochem. Biophys. Res. Commun.* **46**, 2048–2053.
115. Marriott, M. (1980). Inhibition of sterol biosynthesis in *Candida albicans* by imidazole-containing antifungals. *J. Gen. Microbiol.* **117**, 253–255.
116. Alexander, K., M. Akhtar, R.B. Boar, J.F. McGhie, and D.H.R. Barton (1972). The removal of the 32-carbon atom as formic acid in cholesterol biosynthesis. *J. Chem. Soc. Chem. Commun.* 383–385.
117. Aoyama, Y., Y. Yoshida, and R. Sato (1984). Yeast cytochrome P450 catalysing lanosterol 14α-demethylation. II. Lanosterol metabolism by purified P45014DM and by intact microsomes. *J. Biol. Chem.* **259**, 1661–1666.
118. Aoyama, Y., Y. Yoshida, Y. Sonoda, and Y. Sato (1987). Metabolism of 32-hydroxy-24,25-dihydrolanosterol by purified cytochrome P45014DM from yeast: Evidence for the contribution of the cytochrome to the whole process of lanosterol 14α-demethylation. *J. Biol. Chem.* **262**, 1239–1243.
119. Aoyama, Y., Y. Yoshida, Y. Sonoda, and Y. Sato (1989). Deformylation of 32-hydroxy-24,25-dihydrolanosterol by purified cytochrome P45014DM (lanosterol 14α-demethylase) from yeast: Evidence confirming the intermediate step of lanosterol 14α-demethylation. *J. Biol. Chem.* **264**, 18502–18505.
120. Akhtar, M., K. Alexander, R.B. Boar, J.F. McGhie, and D.H.R. Barton (1978). Chemical and enzymic studies on the characterisation of intermediates during the removal of the 14α methyl group in cholesterol biosynthesis. *Biochem. J.* **169**, 449–463.
121. Shyadehi, A.Z., D.C. Lamb, S.L. Kelly, D.E. Kelly, W.H. Schunck, and J.N. Wright *et al.* (1996). The mechanism of the acyl-carbon bond cleavage reaction catalyzed by recombinant sterol 14α-demethylase of *Candida albicans* (other names are: Lanosterol 14 alpha-demethylase, P-45014DM, and CYP51). *J. Biol. Chem.* **271**, 12445–12450.
122. King, D.J., M.R. Azari, and A. Wiseman (1984). Studies on the properties of highly purified cyt. P448 and its dependent activity benzxo(a)pyrene hydroxylase from *S. cerevisiae*. *Xenobiotica* **14**, 187–206.
123. Callen, D.F. and R.M. Philpot (1977). Cytochrome P450 and activation of promutagens in *Saccharomyces cerevisiae*. *Mutat. Res.* **55**, 153–163.
124. Kelly, D. and J.M. Parry (1983). Metabolic activation of cytochrome P450/P448 in the yeast *Saccharomyces cerevisiae*. *Mutat. Res.* **108**, 147–159.
125. Kelly, S.L., D.C. Lamb, B.C. Baldwin, and D.E. Kelly (1993). Benzo[a]pyrene activity in yeast is mediated by P450 other than sterol 14α-demethylase. *Biochem. Biophys. Res. Commun.* **197**, 428–432.
126. Watson, P.F., M.E. Rose, S.W. Ellis, H. England, and S.L. Kelly (1989). Defective sterol C5-6 desaturation and azole resistance: A new hypothesis for the mode of action of azole antifungals. *Biochem. Biophys. Res. Commun.* **164**, 1170–1175.
127. Kelly, S.L., A. Arnoldi, and D.E. Kelly (1993). Molecular genetic analysis of azole antifungal mode of action. *Biochem. Soc. Trans.* **21**, 1034–1038.
128. Bard, M., N.D. Lees, T. Turi, D. Craft, L. Cofrin, R. Barbuch *et al.* (1993). Sterol synthesis and viability of ERG11 (cytochrome P450 lanosterol demethylase) mutations in *Saccharomyces cerevisiae* and *Candida albicans*. *Lipids* **28**, 963–967.
129. Kelly, S.L., D.C. Lamb, D.E. Kelly, J. Loeffler, and H. Einsele (1996). Resistance to fluconazole in *Candida albicans* from AIDS patients involving

cross-resistance to amphotericin. *Lancet* **348**, 1523–1524.

130. Nolte, F.S., T. Parkinson, D.J. Falconer, S. Dix, J. Williams, C. Gilmore *et al.* (1997). Isolation and characterisation of fluconazole and amphotericin B-resistant *Candida albicans* from blood of two patients with leukaemia *Antimicrob. Agents Chemother.* **44**, 196–199.
131. Gachotte, D., C.A. Pierson, N.D. Lees, R. Barbuch, C. Koegel, and M. Bard (1997). A yeast sterol auxotroph (erg25) is rescued by addition of azole antifungals and reduced levels of heme. *Proc. Natl. Acad. Sci. USA*, **94**, 11173–11178.
132. Nes, D.W., G.G. Janssen, F.G. Cromley, M. Kalinowska, and T. Akihisa (1993). The structural requirements of sterols for membrane function in *Saccharomyces cerevisiae*. *Arch. Biochem. Biophys.* **300**, 724–733.
133. Bammert, G.F. and J.M. Fostel (2000). Genome-wide expression patterns in *Saccharomyces cerevisiae*: Comparison of drug treatments and genetic alterations affecting biosynthesis of ergosterol. *Antimicrob. Agents Chemother.* **44**, 1255–1263.
134. De Backer, M.D., T. Ilyina, X. Ma, S. Vandoninck, W.H.M. Luyten, and H. Vanden Bossche (2001). Genomic profiling of the response of *Candida albicans* to itraconazole treatment using a DNA microarray. *Antimicrob. Agents Chemother.* **45**, 1660–1670.
135. Black, S.D. and M.J. Coon (1982). Structural features of liver microsomal NADPH-cytochrome P450 reductase; hydrophobic domain, hydrophilic domain and connecting region. *J. Biol. Chem.* **257**, 5929–5938.
136. Sutter, T.C. and J.C. Loper (1989). Disruption of the *Saccharomyces cerevisiae* gene for NADPH-cytochrome P450 reductase causes increased sensitivity to ketoconazole. *Biochem. Biophys. Res. Commun.* **160**, 1257–1266.
137. Goffeau, A., B.G. Barrell, H. Bussey, R.W. Davis, B. Dujon, H. Feldman *et al.* (1996). Life with 6000 genes. *Science* **274**, 563–567.
138. Venkateswarlu, K., D.C. Lamb, D.E. Kelly, N.J. Manning, and S.L. Kelly (1998). The N-terminal membrane domain of yeast NADPH-cytochrome P450 (CYP) oxidoreductase is not required for catalytic activity in sterol biosynthesis or in reconstitution of CYP activity. *J. Biol. Chem.* **273**, 4492–4496.
139. Lamb, D.C., D.E. Kelly, N.J. Manning, M.A. Kaderbhai, and S.L. Kelly (1999). Biodiversity of the P450 catalytic cycle: Yeast cytochrome $b_5$/NADH cytochrome $b_5$ reductase complex efficiently drives the entire sterol 14-demethylation (CYP51) reaction. *FEBS Lett.* **462**, 283–288.
140. Gonczy, P., C. Eecheverri, K. Oegema, A. Coulson, S.J.M. Jones R.R. Copley *et al.* (2000). Functional genomic analysis of cell division in *C. elegans* using RNAi of genes on chromosome III. *Nature* **408**, 331–336.
141. Shen, A.L., K.A. O'Leary, and C.B. Kasper (2002). Association of multiple developmental defects and embryonic lethality with loss of microsomal NADPH-cytochrome P450 oxidoreductase. *J. Biol. Chem.* **277**, 6536–6541.
142. Lamb, D.C., D.E. Kelly, K. Venkateswarlu, N.J. Manning, H.F.J. Bligh, W.H. Shunck *et al.* (1999). Generation of a complete, soluble and catalytically active sterol 14α-demethylase-reductase complex. *Biochemistry* **38**, 8733–8738.
143. Yoshida, Y. (1988). Cytochrome P450 of fungi: Primary target for azole antifungal agents. In M.R. McGinnis (ed.), *Current topics in mycology,* Springer-Verlag, New York, p. 388.
144. Wilkinson, C.F., K. Hetnarski, and T.O. Yellin (1972). Imidazole derivatives—a new class of microsomal enzyme inhibitors. *Biochem. Pharmacol.* **21**, 3187–3192.
145. Aoyama, Y., Y. Yoshida, S. Hata, T. Nishino, and H. Katsuki (1983). Buthiobate: A potent inhibitor for yeast cytochrome P450 catalysing 14α-demethylation of lanosterol. *Biochem. Biophys. Res. Commun.* **115**, 642–647.
146. Lamb, D.C., D.E. Kelly, M.R. Waterman, M. Stromstedt, D. Rozman, and S.L. Kelly (1999). Characteristics of the heterologously expressed human lanosterol 14α-demethylase (other names: P45014DM, CYP51, P45051) and inhibition of the purified human and *Candida albicans* CYP51 with azole antifungal agents. *Yeast* **15**, 755–763.
147. Lamb, D.C., M. Cannieux, A.G.S. Warrilow, S. Bak, R.A. Kahn and N.J. Manning (2001). Plant sterol 14 α-demethylase affinity for azole fungicides. *Biochem. Biophys. Res. Commun.* **284**, 845–849.
148. Kakeya, H., Y. Miyazaki, H. Miyazaki, K. Nyswaner, B. Grimberg, and J.E. Bennett (2000). Genetic analysis of azole resistance in the Darlington strain of *Candida albicans*. *Antimicrob. Agents Chemother.* **44**, 2985–2990.
149. Venkateswarlu, K., D.W. Denning, N.J. Manning, and S.L. Kelly (1995). Resistance to fluconazole in *Candida albicans* from AIDS patients correlated with reduced intracellular accumulation of the drug. *FEMS Microbiol. Lett.* **131**, 337–341.
150. Sanglard, D., K. Kuchler, F. Ischer, J-L. Pagani, M. Monod and J. Bille (1995). Mechanisms of resistance to azole antifungal agents in *Candida albicans* from AIDS patients involve specific multi-drug transporters. *Antimicrob. Agents Chemother.* **39**, 2378–2386.
151. Sanglard, D., F. Ischer, M. Monod, and J. Bille (1997). Cloning of *Candida albicans* genes conferring resistance to azole antifungal

agents: Characterisation of CDR2, a new multidrug ABC transporter gene. *Microbiology* **143**, 405–416.

152. Sanglard, D., F. Ischer, L. Koymans, and J. Bille (1998). Amino-acid substitutions in the cytochrome P450 lanosterol 14α-demethylase (CYP51A1) from azole resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. *Antimicrob. Agents Chemother.* **42**, 241–253.
153. White, T.C. (1997). Increased mRNA levels of ERG16, CDR and MDR1 correlate with increases in azole resistance in *Candida albicans* isolates from an HIV-infected patient. *Antimicrob. Agents Chemother.* **41**, 1482–1487.
154. Franz, R., M. Ruhnke, D.C. Lamb, S.L. Kelly, and J. Morschhäuser (1998). Multiple molecular mechanisms contribute to a stepwise development of fluconazole resistance in clinical *Candida albicans* strains. *Antimicrob. Agents Chemother.* **42**, 3065–3072.
155. Hamamoto, H., K. Hasegawa, R. Nakaune, Y.J. Lee, Y. Makizumi, K. Akutsu *et al.* (2000). Tandem repeat of a transcriptional enhancer upstream of the sterol 14alpha-demethylase gene (CYP51) in *Penicillium digitatum Appl. Environ. Microbiol.* **66**, 3421–3426.
156. Mellado, E., T.M. Diaz-Guerra, M. Cuenca-Estrella, and J.L. Rodriguez-Tudela (2001). Identification of two different 14α sterol demethylase-related genes (cyp51A and cyp51B) in *Aspergillus fumigatus* and other aspergillus species. *J. Clin. Microbiol.* **39**, 2431–2438.
157. Denning, D.W., K. Venkateswarlu, K.L. Oakley, M.J. Anderson, N.J. Manning, D.A. Stevens *et al.* (1997). Itraconazole resistance in *Aspergillus fumigatus. Antimicrob. Agents Chemother.* **41**, 1364–1368.
158. Ishida, N., Y. Aoyama, R. Hatanaka, Y. Oyama, S. Imajo, M. Ishiguro *et al.* (1988). A single amino acid substitution converts cytochrome $P450_{14DM}$: Complete primary structures deduced from cloned DNAs. *Biochem. Biophys. Res. Commun.* **155**, 317–323.
159. Vanden Bossche, H., P. Marichal, J. Gorens, D. Bellens, H. Moereels, and P. Janssen (1990). Mutation in cytochrome P450 dependent 14α-demethylase results in decreased affinity for azole antifungals. *Biochem. Soc. Trans.* **18**, 56–59.
160. Lamb, D.C., D.E. Kelly, W.H. Schunck, A.Z. Shyadehi, M. Akhtar, D.J. Lowe *et al.* (1997). The mutation T315A in *Candida albicans* sterol 14α-demethylase causes reduced enzyme activity and fluconazole resistance through reduced affinity. *J. Biol. Chem.* **272**, 5682–5688.
161. Loeffler, J., S.L. Kelly, H. Hebart, U. Schumaher, C. LassFlorl, and E. Einsele (1997). Molecular analysis of CYP51 from fluconazole-resistant *Candida albicans* strains. *FEMS Lett.* **151**, 263–268.
162. Lamb, D.C., D.E. Kelly, T.C. White, and S.L. Kelly (2000). The R467K amino acid substitution in *Candida albicans* sterol 14α-demethylase causes drug resistance through reduced affinity. *Antimicrob. Agents Chemother.* **44**, 63–67.
163. Favre, B., M. Didmon, and N.S. Ryder (1999). Multiple amino acid substitutions in lanosterol 14 alpha-demethylase contribute to azole resistance in *Candida albicans. Microbiology* **145**, 2715–2725.
164. Marichal, P., L. Koymans, S. Willemsens, D. Bellens, P. Verhasselt, W. Luyten *et al.* (1999). Contribution of mutations to the cytochrome P450 14 alpha-demethylase (Erg11p, CYP51) to azole resistance in *Candida albicans. Microbiology* **145**, 2701–2713.
165. Perea, S., J.L. Lopez-Ribot, W.R. Kirkpatrick, K. McAtee, R.A. Santillan, M. Martinez, D. Calabrese *et al.* (2001). Prevalence of molecular mechanisms of resistance to azole antifungal agents in *Candida albicans* strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus infected patients. *Antimicrob. Agents Chemother.* **45**, 2676–2684.
166. Boscott, P.E. and G.H. Grant (1994). Modeling cytochrome P450 14-alpha demethylase (*Candida albicans*) from P450CAM. *J. Mol. Graph.* **12**, 185–192.
167. Ji, H.T., W.N. Zhang, Y.J. Zhou, Y.L. Song, J. Lu, and J. Zhu (2000). A three-dimensional model of lanosterol 14 alpha-demethylase of *Candida albicans* and its interaction with azole antifungals. *J. Med. Chem.* **43**, 2495–2505.
168. Delye, C., F. Laigret, and M.F. Corio-Costet (1997). A mutation in the 14α-demethylase gene of *Uncinula necator* that correlates with resistance to a sterol biosynthesis inhibitor. *Appl. Environ. Microbiol.* **63**, 2966–2970.
169. Cosme, J. and E.F. Johnson (2000). Engineering microsomal cytochrome P450 2C5 to be a soluble, monomeric enzyme. *J. Biol. Chem.* **275**, 2545–2553.
170. Williams, P.A., J. Cosme, V. Sridhar, E.F. Johnson and D.E. McCree (2000). Mammalian microsomal cytochrome P450 monooxygenase: Structural adaptations for membrane binding and functional diversity. *Mol. Cell.* **5**, 121–131.
171. Joo, H., Z.L. Lin, and F.H. Arnold (1999). Laboratory evolution of peroxide-mediated cytochrome P450 hydroxylation. *Nature* **399**, 670–673.