Microbial cytochromes P450: biodiversity and biotechnology. Where do cytochromes P450 come from, what do they do and what can they do for us?

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The first eukaryote genome revealed three yeast cytochromes P450 (CYPs), hence the subsequent realization that some microbial fungal genomes encode these proteins in 1 per cent or more of all genes (greater than 100) has been surprising. They are unique biocatalysts undertaking a wide array of stereo- and regio-specific reactions and so hold promise in many applications. Based on ancestral activities that included 14α-demethylation during sterol biosynthesis, it is now seen that CYPs are part of the genes and metabolism of most eukaryotes. In contrast, Archaea and Eubacteria often do not contain CYPs, while those that do are frequently interesting as producers of natural products undertaking their oxidative tailoring. Apart from roles in primary and secondary metabolism, microbial CYPs are actual/potential targets of drugs/agrochemicals and CYP51 in sterol biosynthesis is exhibiting evolution to resistance in the clinic and the field. Other CYP applications include the first industrial biotransformation for corticosteroid production in the 1950s, the diversion into penicillin synthesis in early mutations in fungal strain improvement and bioremediation using bacteria and fungi. The vast untapped resource of orphan CYPs in numerous genomes is being probed and new methods for discovering function and for discovering desired activities are being investigated.

1. Microbial cytochromes P450, sterols and the early metabolism of molecular oxygen

Cytochromes P450 (CYPs) were proposed in the 1970s to have been one of the ways early microbes might have detoxified the first atmospheric oxygen [1], a hypothesis that can lead to much speculation and interest. The observed reactions that CYPs can undertake and that do not require oxygen, obviously points to the possible existence and function of ancestral CYPs before atmospheric molecular oxygen appeared on the Earth about 2.4 Ga. In geological analysis, the presence of sterols that reflect eukaryotic life goes back into the Precambrian and they produce geochemical fossils, steranes, that are detected in rocks as far back as 2.7 Ga [2]. The reliability of these molecules to be as old as the rocks they are contained in has been refuted and the concomitant proof of eukaryotes existing at this point in geological time. The earliest evidence for eukaryotes was revised to 1.78–1.68 Ga [2]. The appearance of oxygen is often called the ‘great oxidation event’, but recently Waldbauer et al. [3] provided evidence that micro-aerobic conditions could have supported steroid biosynthesis before the earliest detectable atmospheric oxygen. An ancestral activity for CYPs is accorded to CYP51 which undertakes an early 14α-demethylation reaction of the first sterol in the pathway, lanosterol, for some micro-organisms and higher eukaryotes; or as in the ‘plant route’ where obtusifoliol (synthesized via cycloartenol) is the CYP51 substrate. Linking this data on the earliest sterols detected and the prerequisite for CYP51 early in sterol biosynthesis, it would seem reasonable to suggest placing the first
CYP51 appearance at 1.78–1.68 Ga according to Rasmussen et al. [2], or earlier. Even if eukaryotes are not as old as this and appeared 850 Ma [4], CYP51 appearance would have been soon after the evolution of the first sterols, as one of the first modifying enzymes of sterol biosynthesis. This would have been in an early eukaryote or possibly a bacterial ancestor. Subsequent to the other steranes detected, further geochemical fossils, 24-isopropylcholestanes, have been used to point to the origin of the Metazoa (marine desmosponges) in rocks to greater than 635 Ma [5].

Sterols were first reported in a bacterium *Methylococcus capsulatus* by Bird et al. [6] and the first bacterial gene (and protein) involved in sterol biosynthesis was identified in this organism, a CYP51 [7]. A route to sterol biosynthesis via lanosterol in this bacterium adds to the discussion on the evolution of sterol biosynthesis that is normally associated with eukaryotes. The *M. capsulatus* pathway may have resulted from horizontal gene transfer, although it is conceivable that a prokaryote ancestor of eukaryotes might have developed sterol biosynthesis [8]. The *M. capsulatus* pathway of sterol biosynthesis contains proteins encoded by overlapping squalene monooxygenase and oxidosqualene cyclase genes that are required before the CYP51 step [8]. Another published pathway of bacterial sterol biosynthesis, leading only to lanosterol and parkeol, was found in *Gemmata obscuriglobus* [9], while in the myxobacterium *Stigmatella aurantiaca*, the production of cycloartenol and the gene responsible was observed [10]. Neither of these diverse bacteria contain a CYP51 activity, but bioinformatic analysis supports the presence of a CYP51 biosynthetic gene and a sterol pathway in another myxobacterium *Plesiocystis pacifica* [11]. Other homologues of sterol biosynthetic genes are detected in bacteria that seem to have been recruited to other functions after gene transfer and for some it is possible to concentrate sterols from the growth media [12].

The sterol pathway requires oxygen at an early point (at squalene epoxidation); this would have been the first step of sterol biosynthesis ‘locking up’ oxygen, but not into sterol. The sterol C4-demethylations and C5-desaturation also add to the extension of the pathway that has been suggested to be driven by oxygen increases [13]. The alternative and not exclusive view is that each addition to the pathway produced a more complex sterol that conferred a selective advantage to micro-organisms regarding their membrane function. The latter is presumably reflected by the addition of a series of reductases to the pathway tailoring the sterol molecule. Subsequent sterol evolution in microorganisms and then multi-cellular organisms presumably led to the varied end products found in some organisms, while for others it led to the loss of sterol biosynthesis entirely since it could be obtained externally, e.g. from the diet as for insects and nematodes.

### 2. Microbial cytochromes P450

The study of microbial cytochromes P450 is of natural academic interest, offering the potential to develop model systems that could assist the understanding of human cytochromes P450 and their role in health. Moreover, the early divergence of microbial CYPs and their extreme biodiversity, which are being revealed through modern genome sequencing techniques, have excited as much interest in their roles in the natural world, as in the consideration of critical applications that may be exploited for future biotechnological needs. As it is now recognized that only a small fraction of microbial life can be cultured, the expansion of metagenomics also contributes in expanding the known diversity of CYPs, such as demonstrated in Craig Venter’s investigations [14]. We hope to reveal here the wide relevance of these genes and proteins for key aspects of what are often called ‘Grand Challenges’, i.e. food security, biorefinery and industrial biotechnology (to create a sustainable future) and for improving and sustaining health.

As in macrobiological systems, CYPs of micro-organisms undertake a wide variety of reaction types (figure 1), the signature of these haemoproteins is to act as mono-oxygenases, but many reactions are reported [15]. These CYPs are key for microbial natural product production, as biocatalysts, for bioremediation and as drug and agrochemical targets.
That cytochrome P450 investigations were dominated in the years after their first identification [16, 17] by mammalian cytochrome P450 was to be expected given the important biosynthetic roles of human forms in, for example, endogenous functions such as steroid biosynthesis and bile acid production, as well as drug/xenobiotic metabolism and its role in pharmacology and toxicology (xenobiotic metabolism). However, cytochrome P450 studies in yeast were initiated during the 1960s [18] and the early expectation was that small numbers of CYPs were present. Not surprisingly therefore, the initial family numbers for lower eukaryotes that were allocated were a modest 51–69, compared with 1–49 for animals. The system for nomenclature of CYP families and subfamilies is dealt with elsewhere in this issue, but is based on amino acid sequence identity (greater than 40% identity placed in same CYP family; greater than 55% in same subfamily). The system was rapidly overwhelmed in the genomic era, with allocated numbers 501–699 soon taken and as the CYP family numbers for lower eukaryotes grew, numbers onwards beyond 5001 have had to be assigned. A single fungal species can often contain more than a hundred CYP genes compared with the 57 found in man and to date more than 2500 DNA sequences have been identified as fungal CYPs.

Many bacteria contain no cytochromes P450; this is true of Eubacteria, for example Escherichia coli, and the Archaea, and early researchers may be forgiven for not anticipating any or few to be discovered. However, bacterial CYPs have also increased substantially in family numbers, with more than 1000 named (CYP101 upwards). The actinomycetes often contain tens of CYP genes in their CYPome and are implicated in the production of many natural products used in medicine (e.g., in streptomyces [19, 20]) and various lipid metabolism in mycobacteria, where CYPs represent potential drug targets [21–23]. Myxobacteria that are sources of natural products such as epothilone anti-cancer drugs are also rich in CYPs [24], but even among actinomycetes some organisms can have low CYP numbers, such as the probiotic organism Bifidobacterium longum can have low CYP numbers, such as the probiotic organism Bifidobacterium longum that has no CYPs. Microbial CYPs have revealed diverse classes [36] that will be useful in biotechnology. Ten classes have currently been assigned. Most bacterial CYPs are soluble and are so-called Class I and driven by ferredoxin and ferredoxin reductases. There are often many genes for both these latter proteins encoded in bacterial genomes and few complete studies on functional redundancy have been performed such as for Streptomyces coelicolor A3(2) CYP105D5 [37]. This class also includes the archetypal CYP101 or P450CAM from P. putida described above.

Class II includes most eukaryote CYPs localized to the endoplasmic reticulum and some other membranes via an N-terminal anchor and associated with a separate NADPH-cytochrome P450 reductase (CPR). This is not the only electron donor in microbial Class II systems as in yeast a knockout of the gene is still viable and continues to show CYP51 activity in sterol biosynthesis that is required for viability [38, 39]. The cytochrome $b_5$ system has been attributed with this sustained activity since a double knockout is lethal. Other roles of yeast CPR have been revealed in cell division giving rise to polarity defects indicating a role for CPR in bud site selection, bud growth, cell wall assembly, mating and invasive growth [40, 41]. The structure of yeast CPR has been determined in a form that retains the ability to drive CYP activity, the only such example to date [42]. CPR is usually encoded by only one gene in most eukaryotes except plants [43] and some filamentous fungi [44]. The roles of these multiple CPRs is of clear interest and in aspargilli and some other fungi is also accompanied by duplicated CYPs, e.g., CYP51 that impinges on antifungal therapy with CYP51 azole drug inhibitors [44–46]. In Cochliobolus lunatus, the cytochrome P450 system is of biotechnological interest as the fungus is used in preparation of corticosteroids and CYPs have been associated with detoxification of plant defence compounds [47]. Lah et al. [48] reported two CPRs in this fungus where one was associated with primary metabolism and the other with xenobiotic detoxification. Interestingly, when reconstituted with a CYP53 the two CPRs produced different products suggesting another level of control of cytochrome P450 was implicated in the growth of Candida tropicalis for this purpose, where it undertook the initial metabolism of these carbon sources [33]. Biotechnological applications were also discovered subsequently to involve CYPs, such as for some of the first industrial biotransformations in the 1950s undertaken by filamentous fungi in the production of corticosteroids by using their abilities to undertake steroid 11-hydroxylation, reactions difficult to achieve chemically [34]. More recently, it was realized that mutation in CYP504 was responsible for channelling metabolites into penicillin production, instead of 2-hydroxylation of phenyl acetate, during some of the earliest titre improvements in the Wisconsin strains by industrial strain improvement [35]. With the identification of CYP sequences comes the challenge of identifying protein function, and it is of interest that an increasing number of microbial CYPs are being associated with secondary metabolic pathways undertaking oxidative decoration of natural products that may be exploited in fine chemical production.
metabolism and presumably an effect of CPR binding on the CYP substrate bound structure.

Class III CYPs are to date bacterial in origin and use flavin mononucleotide (FMN) containing flavodoxin as the reduction partner, first found for CYP176A1 (P450cin) metabolizing cineol [49]. Class IV is also of microbial origin found in an acidothermophilic archaeon. CYP119 in *Sulfolobus solfataricus* can obtain electrons from a non-NAD(P)H-dependent reductase [50] and is active at high temperature and pressure. Class V CYPs are represented currently by CYP51 from *M. capsulatus* and contains a C-terminal fusion via a short linker to a (3Fe-4S) ferredoxin domain (figure 2). This CYP was the first bacterial sterol biosynthetic gene/protein reported [7] and was discussed above.

CYP177A1 is another new class of cytochrome P450 (Class VI) encoding a natural fusion between an N-terminal flavodoxin domain and a C-terminal haem domain identified by Seth-Smith *et al.* [51]. It was identified in *Rhodococcus rhodochrous* (strain 11Y) and it allows this bacterium to obtain nitrogen from a high-explosive pollutant called RDX (see earlier). Class VII CYPs have been reported as natural fusions of CYP116B2, also called P450 Rhf, from *Rhodococcus* sp. NCIMB 9784. This protein consists of a C-terminal reductase domain containing FMN and NADPH binding motifs and a ferredoxin-like domain with a N-terminal haem linked to the C-terminus via a linker [52,53].

Class VIII CYP systems consist of an N-terminal haem domain fused to a eukaryotic-like CPR-like domain at the C-terminus. The first example emerged from the Fulco laboratory for CYP102A1 (P450BM-3), a protein from *Bacillus megaterium* that metabolizes fatty acids [54]. A similar membrane-bound fungal system was also discovered in *Fusarium oxysporum* CYP505 [55] and subsequently in a number of filamentous fungal genomes [45]. Class IX CYP was assigned to CYP55 (P450nor) also from *F. oxysporum* and identified in the Shoun laboratory. This CYP is a nitric oxide reductase and a soluble eukaryotic CYP that derives electrons from NADH [56]. The last CYP class X assigned to date includes CYPs that catalyse reactions without a redox partner, first associated with plant allene oxide synthase [57]. The reactions of this class that includes animal and plant CYPs involve an intramolecular transfer system and examples include divinyl ether synthase and thromboxane synthase [36,57].

Without doubt the number of classes will increase from the current knowledge of the ‘tip of the iceberg’ of metagenomic and culturable microbial genomes. Bacterial CYPs exhibiting peroxygenase activities have been discovered that bind *H₂O₂* with high affinity and turn over fatty acid at a high rate; the first identified was CYP152A1 from *Bacillus subtilis* [58]. These could comprise an additional class and they seem not to require a reduction system for oxygen activation. In the *Aspergillus nidulans* CYPome, the identification of PpoA (CYP6001A1) as a fusion protein of a fatty acid dioxygenase/peroxidase and a cytochrome P450 was made after characterization of the heterologous protein produced in *E. coli*. It displayed the characteristic 450 nm carbon monoxide binding spectrum and is involved in regulating the balance of sexual and asexual life cycles by metabolizing fatty acids to...
produce precocious sexual inducer factors [59]. Another cytochrome P450 has also been found recently with more than one activity, but not necessarily reflecting a different CYP class. CYP170 was identified as possessing a hydroxylase activity participating in albaflavenone biosynthesis in S. coelicolor A3(2), as well as having a separate farnesene biosynthetic terpene synthase active site [60].

4. Microbial cytochromes P450 and the environment

Some of the largest numbers of CYPs in individual microbes are found in white- and brown-rot fungi. In Phanerochaete chrysosporium, about 150 CYPs were identified thus revealing the scale of unravelling orphan CYP function that faces the community, as only a few have defined functions or (useful) activities associated with them (e.g. CYP51, [61]). The detection of cytochrome P450 in this fungus and demonstration of catalytic activity in the metabolism of poly cyclic aromatic hydrocarbons [62] has been followed by examination of CYP gene expression patterns upon exposure to an inducer of this class of compound, to reveal those CYPs with this functional profile [63]. This fungus is of further interest with respect to commercial bioremediation and the fungus’ ability to degrade lignin with breakdown also associated with CYP activities [64,65]. These CYP activities are less well investigated than the extracellular peroxidases and laccases involved in this process. The importance of CYPs in the breakdown of plant material in the environment in white-rot fungi is true also of brown-rot fungi [66] where an even larger number of CYP genes have been discovered [67]. Two hundred and fifty CYP genes were identified in the Postia placenta genome of which 184 were isolated as full-length cDNA and expressed in S. cerevisiae to probe activity that included stilbene as a CYP substrate.

The large biodiversity of CYPs is not only found in these wood-rot fungi, but in other saprophytic organisms, e.g. Aspergillus oryzae [68], and in soil actinomycetes such as the 18 CYPs in S. coelicolor A3(2) [19] and approximately 40 in Mycobacterium smegmatis [12,69]. The ability of these organisms to biotransform xenobiotics is well known, including for instance, the ability of aspergilli in industrial steroid hydroxylation. As for some human CYPs that have evolved to assist detoxification of natural products in the diet and which now are known to contribute centrally to drug metabolism, some of these microbial CYPs have the capability to metabolize drugs and have been used to produce mammalian drug metabolites. These metabolites have been needed for pharmacological and toxicological evaluation in the drug discovery process particularly in the days before heterologous human cytochromes P450 were available commercially.

Some microbial CYPs may have been engaged in detoxification as well as synthesis of natural products and in microbial systems of fungi and bacteria CYPs can contribute to bioremediation. For example, one study examined Rhodococcus and Gordonia strains for the ability to degrade petrol additives methyl tert-butyl ether, ethyl tert-butyl ether and tert-amyl butyl ether and found an involvement of CYP249 [70].

Another very important role of fungal CYPs in the cycling of nutrients includes CYP55 of fungi, a fungal de-nitrification enzyme contributing to the nitrogen cycle [71]. De-nitrification is a process where nitrate or nitrite is reduced to a gaseous form of nitrogen (N₂ or N₂O) and fungi lacking nitrous oxide reductase produce nitrous oxide, a greenhouse gas, as a final product [72]. Studies have revealed fungi are a major contributor to de-nitrification in grasslands, semi-arid and forest soils with implications for soil fertility [73].

Dependent upon the ecological niche new and interesting roles for CYPs have been discovered, for example, insects that ‘farm’ fungi to provide a source of sterol [74]. CYPs also play a part in fungal pathogenicity against insects. The insect cuticle comprises a mixture of lipids, including long-chain alkanes, alkenes, wax esters and fatty acids, and recently the involvement of CYPs in breaking down this barrier was shown for Beauveria bassiana [75]. Eight CYPs from seven different CYP families were associated with metabolism of cuticle lipid. These included CYP52s, originally identified as alkanes and fatty acid metabolizing CYPs in Candida spp. [33], but also a member of a new family of CYPs, CYP5337, as well as new members of subfamilies from the CYP53 family. Candida spp. contain multiple CYP52s with overlapping substrate specificity shown by biochemical and genetic methods [76,77], and it was suggested that the CYPs identified as being induced by cuticle lipids would also show such overlap [75]. Manipulating these activities could increase virulence and has the potential to assist in bio-control of insects using fungal pathogens.

5. Food security and microbial cytochromes P450

Microbial CYPs have been revealed to be important for food security issues and one of the main reasons is that many fungal pathogens of crops are controlled by inhibitors of CYP51 that block ergosterol biosynthesis [78,79]. These were first introduced in the early 1970s. About 40 per cent of the agrochemical market is for fungicides and to maintain and increase yields will require their continued use for the foreseeable future. There have been concerted screens to develop new fungicides but, as in the pharmaceutical area, this has led to limited success. The strobilurin fungicides were successfully introduced and target the respiration pathway by inhibiting cytochrome bc1, but several plant pathogens have developed resistance reaching high levels [80]. The continued success of CYP51 inhibitors (also known as azole fungicides or demethylase inhibitors) has led to renewed interest in discovery of new agents also set against a backdrop of European regulation to improve the safety of pesticides. The azole antifungals represented an older era of drug and agrochemical discovery. Although they were introduced without knowledge of their mode of action in the 1970s, it was later that they were recognized to inhibit firstly sterol biosynthesis among other cellular effects [78] and then more specifically, fungal cytochrome P450 undertaking sterol 14α-demethylation [81]. They are central to antifungal therapy clinically, where the immuno-compromised are at risk, and despite attempts to discover better agents are central to control of many plant pathogens. In both the clinic and the field, the emergence of resistance is a problem.

The gene encoding the particular target emerged from studies on Saccharomyces cerevisiae and was named CYP51 in the nomenclature [82]. It was found to be an essential gene for viability, a result confirming it as the target after the various biochemical correlations suggesting this. We now appreciate this CYP activity is ancient and other CYP51s are found in...
sterol-producing animals, plants, protists, but rarely in bacteria, producing 14α-demethylated sterols [69,83]. The 14α-demethylation involves three sequential monooxygenase events with release of formic acid [84–86].

As proposed in 1995 [87], another cytochrome P450 CYP61 (sterol 22-desaturase) represents an ancient activity for the superfamily being present as CYP710 in plants [88]. Homologues in lower eukaryotes have also been assigned CYP710 status, although they often make the ‘fungal sterol’ ergosterol. These two CYP families should be unified, as is the case for CYP51s with a conserved function. One of the first uses of amino acid sequence information to assign function to a gene in a newly sequenced genome was made for CYP61 and the suggestion that CYP61 arose from CYP51 during the successive addition to the sterol biosynthetic pathway during its evolution in unicellular organisms. Subsequently, in 1997, the activity and sensitivity to azole drugs was characterized [87–90]. We wait to see whether CYP61/CYP710 emerges in sponge genomes, as it is present in the choanoflagellates (figure 3), ancestors of fungi and animals [91], and sponges have been reported to contain 22-desaturated sterols [92] that require CYP61/CYP710.

To date, it has not been found in the first sponge genome to be sequenced from Amphimedon queenslandia (D. Nelson 2011, personal communication).

The genes encoding the enzymes that undertake the ancient CYP51 activity are undergoing evolution in the field as a response to use of azole inhibitors that bind to this target. Even with resistance, crop management can be maintained by altering fungicides and using mixtures. Azole inhibitors of CYP51 act by binding via the N-3 of imidazole or N-4 of triazole to the haem as a sixth ligand, while the N-1 substituent group binds to the amino acid tertiary structure of the active site and substrate channel (figure 4) [20]. To date, there are no fungal CYP51 structures, although the structure of human CYP51 was recently elucidated [93].

The decreased sensitivity of the wheat pathogen Mycosphaerella graminicola to azole fungicides appears to be mainly due to mutation in CYP51 and this is true for other important plant pathogens. A complex and increasing number of mutations have been revealed over time within the same gene, indeed it is now impossible to find wild-type CYP51s in the field in the UK. Yeast complementation tests revealed these mutations resulted in functional, but resistant, protein activity [94]. The substitutions L50S, Y459D and Y461H and a two-amino-acid deletion mutant Delta Y459/G460 all complemented yeast with their activity and conferred resistance. Another change, I381V, inactivated protein activity, but this was restored when found in combination with other mutations at Y459/Y461 [94]. Mutation at S524T was also recently shown to confer resistance and to rescue activity in combination with Y137F and V136A mutations. Combinations of recent alterations detected in the field of D134G, V136A, Y461S and S524T also reduced sensitivity to the two main commercial fungicides currently used, epoxiconazole and prothioconazole [95].

A structural rationale has recently emerged for these CYP51 resistance mutations using a multiple homology method, individually identified for each variant, rather than a single structural scaffold. Using this approach, the development of resistance could be explained when considering multiple mutant CYP51s, through modified structure and increases in the binding pocket volume, while also explaining the regained sensitivity to some earlier fungicides in some recent mutants studied using yeast complementation [94,95]. This approach provides a potential tool to manage
fungicide use and explore potential for resistance for new fungicides [96].

The participation of CYPs in biosynthetic pathways of natural product synthesis can, in the context of food security, be a problem because of the synthesis of mycotoxins. Mycotoxins are synthesized by many phytopathogenic and food spoilage fungi, including Aspergillus, Fusarium and Penicillium spp. These can have significant health effects: the carcinogenic effect of aflatoxin, and nuts and grains need to be monitored for mycotoxins. For example, two

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Apart from mycotoxin biosynthetic pathways, ergot fungi are also well known for their effects on contaminating food causing ergotism after, for example, the consumption of contaminated rye bread. The pathway includes a CYP required for the conversion of clavines to D-lysergic acid among a cluster of ergot alkaloid genes [101]. Mindful of Paracelsus’ famous quote, these ‘poisons’ have also been turned to pharmaceutical use in migraine treatment and in inducing contractions in the uterus to stop bleeding after childbirth.

Bacterial CYPs may also be important in aspects of food security, but that is less clear than for the fungal examples described. Streptomyces scabies causes potato scab disease; the completed genome has multiple CYPs and their potential roles in pathogenicity and as targets of agrochemicals deserves attention. The main phytotoxin produced by S. scabies is thaxtomin and biosynthesis of thaxtomin cyclic dipeptide phytotoxins requires CYP for postcyclization hydroxylation of the cyclic dipeptide [102].

Beyond bacteria and fungi, there are other important microbial pathogens, including the fungal-like oomycetes that have filamentous growth. The potato blight and sudden oak death pathogens are examples of Phytophthoras that are organisms that do not synthesize sterols, but alternative CYPs are potential targets [103]. The soybean pathogen P. sojae has a CYPome of 30 CYPs, while the sudden oak death pathogen P. ramorum contains 24 CYPs. Other examples of oomycete pathogens include the legume pathogen Aphanomyces euteiches, which does have a sterol biosynthetic pathway, including a CYP51, the proven fungicide target [104]. The suggestion in this paper that similar products to theazole fungicides could be introduced for the Saprolegniales would also be useful in fish farming where

Figure 4. (a) A variety of azole structures, including clotrimazole, fluconazole, voriconazole and itraconazole (medical antifungal drugs) and the agrochemical demethylase inhibitors prochloraz, triadimenol, epoxiconazole and prothioconazole. (b) The molecular model of triadimenol bound to M. graminicola CYP51 with locations of resistance residues Y137 and I381. (c) Ergosterol biosynthesis from lanosterol highlighting the 14α-methyl group removed by CYP51.
these pathogens are problematic and dealt with, until recently, using toxic malachite green.

6. Industrial biotechnology and a sustainable future

Many of the topics and themes running through this paper and volume overlap and reference to important developments in industrial biotechnology already touched on. Hence the initial importance of CYPs in biotransformations was achieved using fungal CYP activities in whole-cell biotransformations to allow corticosteroids to be produced. The impetus for this may have been in the belief that the Luftwaffe experimented with these compounds and their derivatives to enhance pilot performance in the Second World War [105] stimulating organic chemistry research in a manner similar to the push for penicillin production, via fermentation and strain improvement, in the development of biotechnology. The regio- and stereo-specificity in steroid biotransformation by individual fungal species varied, presumably reflecting the CYPs concerned [34]. Despite this historical importance, only recently has a fungal steroid hydroxylase been identified from Rhizopus oryzae [106]. As in aspergilli, the 11α-hydroxylation of the steroid skeleton can simplify steroid drug production. CYP509C12 was identified among 48 putative CYPs as being inducible by addition of progesterone, and its activity confirmed by heterologous expression in fission yeast. This could lead the way for altered methods in the preparation of steroids using alternative microbes or enzymes.

One major example of such an alternative approach was the metabolic pathway engineering of the ergosterol pathway in S. cerevisiae to divert it towards corticosteroid production [107]. This required manipulation of activities other than CYPs and the yeast pathway was diverted by expression of a plant and eight mammalian proteins, including CYP11A1, CYP11B1, CYP17A1 and CYP21A1. A recent paper also discusses the alteration of yeast to a form producing cholesterol efficiently instead of ergosterol [108]. Other ambitious examples of pathway engineering that required CYP as an important player are the production of the anti-malarial drug precursor artemisinic acid using yeast [109] and taxol precursor in E. coli [110].

The promise of cytochromes P450 in industrial biotechnology extends to bioremediation purposes described earlier and in chemical production using whole cells. Statin biotransformation has also been used to modify a fungal natural product to produce a major drug. Mevastatin is produced by Penicillium citrinum and hydroxylation of mevastatin by Streptomyces carboxophilus is used to produce the anti-cholesterol drug pravastatin [111].

Another current application of whole-cell biotransformations is for the production of dicarboxylic acids from fatty acids and alkanes. These chemicals are versatile intermediates that could be used to produce polymers, to replace oil-based products in a bio-refinery model and lead to further high-value end products. The ability for CYP52A3 to undertake the reaction cascade to dicarboxylic acids was first reported in the 1990s [112]. A variety of Candida spp. are known to have CYP52 family gene sequences, but not all CYP52s produce diacid. The ability of C. tropicalis to produce α,ω-dicarboxylic acid was enhanced by blocking β-oxidation so that these products were not used for growth, and this has formed the basis of an industrial process [113]. CYP52 in Candida spp. can also have involvement and potential application in the production of sphorolipids that have diverse applications, including as surfactants [114].

The advantage of whole-cell biotransformation is the presence of a cellular system to generate electrons for these reactions and the presence of the CPR system needed to deliver these to CYPs. An alternative to this is to use purified proteins in a reconstituted system, but the main issue currently is the value of the product set against the cost of the process and, most importantly, knowledge of the specific biotransformation for each CYP. Indeed, whole-cell CYPome analysis using heterologous gene expression in yeast has been used to screen for activities across hundreds of fungal CYPs [82]. These studies used yeast expression with co-expression of CPR. In a similar study on a smaller CYPome from the actinomycete Nocardia farcinica, a novel high-throughput ligation independent system was developed for E. coli in which the CYPs were fused to a reductase domain of the fusion protein of CYP116B (P450RhF) and for which approximately half the number of CYPs could be screened subsequently and yielded activities [115]. It is rare that all CYPs examined can be expressed well in high-throughput on the first pass, and subsequent investigations may be needed to achieve high-level expression for the recalcitrant CYPs. High-throughput methods are needed to reveal orphan CYP activities in genomes and metagenomes for biotechnology that will augment the green biotechnology approaches that are emerging. Such an approach will also be important for revealing the endogenous functions of orphan CYPs. The main alternative method currently to identifying endogenous activity and function of CYPs is the metabolomic comparison of wild-type and mutant microbes where radioisotopic comparisons may assist. For example, for CYP154A1 from S. coelicolor, the substrate was identified in this way as a dipentaenone and then shown to be metabolized by CYP154A1 via an intra molecular cyclization without oxidation/reduction in a novel reaction for CYPs [116].

The main area where purified CYPs are used commercially and produced as biotechnology reagents is in toxicology related to chemicals, agrochemicals and pharmaceuticals. Here the fate of xenobiotics with respect to human (mammalian) CYP metabolism is investigated, in part, as a registration requirement for the use of the drugs/compounds and also as a determination of those metabolites activities in relation to parent substrates. In other settings besides drug metabolism, e.g. for flavours and fragrances, CYPs could enhance properties and add value [117]. The metabolic fate of xenobiotics is revealed for the main CYPs undertaking metabolism, and the metabolites can be prepared for further studies. These CYPs and CPR are produced in microbial systems usually, and it is common in E. coli systems to modify the N-terminus to achieve heterologous protein expression [118], with and without the membrane anchor [119].

For other industrial applications, the supply of pure enantiomers as substrates and the use of expensive cofactors, or cofactor recycling, may not justify the use of pure enzymes currently, but this is an active area of investigation. Feasibility studies on immobilizing CYP systems have been published using purified CYP and colloidal liquid aphrons [120] and mammalian membrane-bound CYPs have been incorporated
into nanodiscs together with CPR to investigate activity/ application [121]. Cofactor recycling has also been investigated in experiments using CYP105D1 [122], and activity in biotransformations has also been studied using CYP activities driven by electrodes. Cobalt (III) sephalurate was used as a mediator and platinum electrodes allowed activity of CYPs to be achieved albeit with reduced activity [123,124].

Of course, genetic manipulation and process engineering can be used to enhance whole-cell biocatalysts as well as enzymes. Cell walls and membranes and endogenous transporters can provide barriers, while product purification from a whole cell can present process issues when compared with cleaner single enzyme systems. Whether using whole cells or pure enzymes, the improvement of activity using directed evolution has also been applied to CYPs, and CYP102A1 or P450BM3 has been a favourite for this as it has a turnover much higher than typical eukaryotic CYPs. Instead of values around 1, often reported for mammalian CYPs, it can turn over arachidonic acid at 17 100 min⁻¹ [125]. This is presumably because it is very efficiently coupled to the reductase as a fusion protein. Early directed evolution experiments altered P450 for more efficient use of peroxide to support activity, including for P450BM3 [126,127]. Subsequent experiments altered P450BM3 [128] to include metabolism of the gaseous alkanes propane and butane. CYP156A6 from Mycobacterium sp. strain HXN-1500 has also been improved for terminal butane oxidation [129]. The abundance of methane could be used to replace petroleum as a feedstock for industry and can also be produced sustainably by anaerobic digestion from biomass. It is interesting to speculate that CYPs might be developed to help achieve that goal. If not, these methods will still be used as routes to drug preparations, fine chemicals, biorefinery and bioremediation in a manner that will contribute towards a sustainable future.

The identification of lipids and fatty acids as CYP substrates has been a feature of this and many publications on the superfamily. Recently, a CYP152 was identified as a novel fatty acid decarboxylase from Jetogallus sp. ATCC 8456 [130]. The ability of this CYP to produce 1-alkenes from fatty acids has enormous potential for fuel and chemical feedstock production and is being exploited by LS9 Inc. in California, who discovered this property. Other CYP152s from other bacteria are highly active with hydrogen peroxide and this was true of the fatty acid decarboxylase discovered.

### 7. Microbes, cytochromes P450 and human health

Microbial CYPs are intimately associated with many of the natural products used as medicines. By metabolic engineering and in future synthetic biology they will be incorporated into strategies to produce, or enhance, known and new drugs sustainably using microbes [109,110]. Among bacterial CYPs probably the earliest antibiotic biosynthesis CYP gene and protein studied in detail was P450 erF (CYP107A1) involved in C6-hydroxylation of the macrolide 6-deoxyerythronolide B during erythromycin biosynthesis in the bacterium Saccharopolyspora erythraea [131,132]. As mentioned above, many pathways of bacterial and fungal secondary metabolism contain CYPs involved in multi-step oxidation, rearrangements, epoxidations and heteroatom oxidation. Microbial CYPs can contribute other activities in secondary metabolism, such as carbon–carbon bond formation in flavin polymer biosynthesis involving CYP158 [133]. These diverse reactions are important for the activity of the final compounds and their diversity (table 1).

Streptomycetes produce about 70 per cent of the antibiotics by type, although in market value the fungal penicillin and cephalosporin antibiotics are dominant. The CYPome of S. coelicolor A3(2) revealed 18 CYPs and, as in other genomes subsequently, unsuspected secondary metabolism pathways involving orphan CYPs often organized as operons [33]. In the case of CYP158A2, a three-gene operon was identified encoding a Type III polyketide synthase. In other examples, CYPs of S. coelicolor A3(2) appeared in operons with a non-ribosomal peptide synthase (CYP105N1, 33) and another (CYP170A1) adjacent to a terpene synthase, subsequently determined to be involved in albaflavanone biosynthesis [134]. The operons can often provide clues to enzyme activities and by combining experimental approaches, e.g. gene knockouts, expressing whole pathways, or purifying individual enzymes to produce substrates for testing, may thereby reveal function of individual proteins. More problematic are those CYPs not associated with operons and at loci that give no clue to function, and a number of S. coelicolor CYP functions remain to be determined.

Antibacterials other than erythromycin also require CYPs for biosynthesis, including vancomycin, important in combating antimicrobial resistance. CYP146 is the fourth gene in the vancomycin gene cluster from Amycolatopsis mediterranei and is a carrier-protein binding CYP involved in β-hydroxytyrosine formation [135]. In addition to antibacterials are metabolites requiring CYP for their synthesis and which target other pathogens, such as for the anthelmintic avermectin synthesis in S. avermitilis [136]. The polyene antifungals nystatin from Streptomyces noursei and amphotericin B from Streptomyces nodosus also require CYP for their biosynthesis. Gene knockouts of the CYP in S. nodosus altered the polye produced and deleting/adding CYPs could produce new antibiotic types [137]. Similar studies on two cytochromes P450s, NysL and NysN, from S. noursei produced new analogues [138]. CYPs are also involved in anti-neoplastic natural product synthesis, such as in the actinomycin D gene cluster in Streptomyces chrysomallus [139]. Other bacteria, such as the myxobacterium, produce natural products and EpoK, CYP167A1 is involved in biosynthesis of the promising anti-cancer epithilones A and B [140].

Fungal secondary metabolism frequently has CYPs involved and these may occur in gene clusters associated with polyketide synthases [44] and, for example, lovastatin biosynthesis in Aspergillus terreus [141]. Lovastatin is a simvastatin precursor that is a semi-synthetic cholesterol-lowering agent. The loxA gene encodes a cytochrome P450 clustered with the lovB polyketide synthase. LovA performed two previously elusive oxidative reactions, introduction of a 4α,5-double bond and C-8 hydroxylation. An interesting experimental development in an investigation of programming new polyketide synthases, coupled to co-expression of two CYPs, led to the resurrection of an extinct metabolite, basianin, in Aspergillus oryzae [142]. Future manipulations of microbes may allow new secondary products to be produced in a manner analogous to the streptomycete and fungal examples above.

Apart from the health benefits CYPs provide in terms of naturally occurring drugs and platform chemicals for future
pharmaceuticals, the microbial CYPs have proved to be drug targets themselves, via the CYP51 antifungal inhibitors [69,143,144]. As mentioned earlier, the azole antifungals are a main plank of therapy in the clinic with many products also available over the counter in pharmacies. Orally activeazole antifungals began to be introduced with ketoconazole in the 1980s after previous topical agents had been commercialized, such as econazole, miconazole and clotrimazole. Owing to toxicity issues and because of drug–drug interactions with drug metabolizing CYPs that can disturb the clearance of drugs, better agents were required. Fluconazole offered better safety and higher solubility by the late 1980s.

Concurrent with this antifungal drug discovery the HIV pandemic was emerging. Candidiasis in HIV patients was often the first presentation of their condition and, together with prophylactic azole treatment over years, saw the first serious emergence of clinical antifungal drug resistance (figure 5) [145,146]. Fluconazole was not very effective in managing another fungal disease, aspergillosis, but itraconazole and voriconazole were shown to be useful later. Itraconazole resistance in aspergillosis emerged during the 1990s [147] and with it the possibility of the interplay between exposure to agrochemical azoles acting as a factor in resistance arising in the clinic [148]. There is a difference between concerns about the spread of resistance in fungi from that in bacteria, as the mechanisms in fungi involve chromosomal genes and do not involve highly transmissible plasmids. Newer azoles have come through to the clinic, such as posaconazole, and in the absence of alternative targets to CYP51 being found, further azole compounds are coming into clinical trial. Prior to azoles, the therapy used was amphotericin B-based which can lead to renal problems if prolonged. For topical use, nystatin was available. Alternative β-glucan synthase inhibitors have also emerged as drugs, e.g. caspofungin and micafungin [149].

Fungal infection has been increasing due to the rise of immuno-compromised conditions such as chemotherapy, organ transplantation and HIV infection [143]. Currently, the market is predicted to grow to $8.4 billion by 2016. The current top three diseases requiring antifungal therapy by value are posaconazole, and in the absence of alternative targets to CYP51 being found, further azole compounds are coming into clinical trial. Prior to azoles, the therapy used was amphotericin B-based which can lead to renal problems if prolonged. For topical use, nystatin was available. Alternative β-glucan synthase inhibitors have also emerged as drugs, e.g. caspofungin and micafungin [149].

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antifungal market voriconazole sells $820 M per annum, caspofungin $600 M per annum, liposomal amphotericin B (ambisome) $500 M per annum, with posaconazole and micafungin at $500 M per annum when combined (http://www.aspergillus.org.uk/updates/NewsletterSeptember2011.html). Another important consumer niche for azole antifungals is the aspergillus.org.uk/updates/NewsletterSeptember2011.html). A. fumigatus... of residues associated with fluconazole resistance in CYP51 in C. albicans, the different amino acids associated with resistance in A. fumigatus and those seen in plant pathogens [69]. Obviously, the different CYP51 proteins and the different azoles they are exposed to, together with different environments, will select different molecular events. For C. albicans, new mutations from the clinic were observed in the late 1990s and by genetic screens in yeast transformants these were associated with resistance [153–155] as well as observations of the emergence of resistance in matched sets of sequential isolates from patients [153].

After demonstration that R467K was a substitution in C. albicans causing fluconazole resistance [153], a number of other mutations were observed in resistant strains and placed in an early molecular model for CYP51 [154]. The mutations included another apart from R467K, namely G464S, that is also part of the haem binding domain and which might cause resistance by altering the position of the bound azole above the haem. These mutations were observed to cause resistance in yeast transformants together with another common mutation Y132H [155] and S405F. As in azole-resistant plant pathogens some isolates also contained combinations of mutations. These were often in a homoygous state in this diploid pathogen suggesting gene conversion had occurred in the second allele conferring higher resistance. Biochemical analysis has provided support for the altered affinity of the mutant proteins forazole drugs such as for G464S [156] and more recently I471T, an amino acid adjacent to the cysteine at 470 that acts as a haem ligand. In the case of I471T, the resistance was attributed to a combination of increased turnover, increased affinity for substrate and a reduced affinity for fluconazole in the presence of substrate allowing the enzyme to remain active at higher fluconazole concentrations [157].

The molecular basis of resistance and in turn the best therapeutic response to it are still under investigation. Shortly after the outset of fluconazole resistance in C. albicans being studied, it was realized that not only diverse point mutations in CYP51 were involved in resistance, but also in multiple mechanisms [158,159]. This is not yet clear for resistance in filamentous fungi. The other mechanisms clearly identified in C. albicans are increased expression of drug transporters, particularly CDR1, through mutation in a transcription factor [160] and altering the pathway of 14α-demethylated sterols accumulating under treatment away from a toxic 14α-methyl-3,6-diol end product to 14α-methylcholesterol that C. albicans can grow on [161,162]. This latter mechanism involves suppression of the effect of azoles through mutation in the sterol C5-desaturase (Erg3). It is presumed the enzyme undertakes a faulty desaturation producing the 6-OH group of the 14-methylated sterol. The sterol 6-OH of the 14α-methyl-3,6-diol is proposed to interfere with phospholipid interactions in membrane function. Azole resistance of this type has been referred to as infinite resistance. Although the literature refers frequently to the accumulation of 14-methylated sterols being the cause of arrest under azole treatment this is contrary to the concept of the value of sterols, and oxidosqualene cyclase, to organisms when sterols evolved to allow a primitive sterol pathway [162]. Not all 14α-methyl sterols are inconsistent with growth and genetic studies indicate lanosterol supports growth in yeast [163]; we have found recently a Candida glabrata that grows with lanosterol as a main sterol (SL Kelly and DE Kelly 2011, unpublished observation).
Other mechanisms of resistance have been observed in *C. albicans*, including increased transcription of CYP51, but this has been examined by heterologous hyper-overexpression and only changes resistance by a small amount compared with the several orders of magnitude increase in CYP51 [144]. Aneuploidy and isochromosome formation in the locus of CYP51 have been associated with resistance [164], but small changes in CYP51 expression itself may not confer much resistance as between haploid and diploid [165], but small changes in CYP51 expression itself may not confer much resistance as between haploid and diploid *S. cerevisiae*. Studies in other fungi have mainly revealed resistance mechanisms associated with altered CYP51 and in *A. fumigatus* these are found in two CYP51s in the genome. Both CYP51A and CYP51B were shown by yeast complementation to function in 14α-demethylation [46], but only CYP51A and not CYP51B exhibits alterations associated withazole resistance [148]. Together with protein affinity studies it appears the CYP51A is most resistant inherently and therefore selection pressure is exerted on this form [45]. However, it would appear unlikely that CYP51B was unable to become resistant in any scenario and this requires further investigation. The mutations observed in CYP51 associated with resistance are at positions not found in *C. albicans* and include G54, L98 and M220 [148]. Other mutations have been observed, for instance G138C, Y431C and G434C and also increased CYP51A expression in pan-azole-resistant *A. fumigatus* [165].

In other microbial pathogens, CYPs may in future be targeted to treat patients. Lessons from resistance to antifungals and more CYP51 and other protein structures to inform drug development will be useful. CYP51 is the target in fluconazole therapy of *Acanthamoeba polyphaga* causing eye infections associated with contact lens use [166]. Amoebae can also cause serious disease in the immuno-compromised and harmful bacteria, such as methicillin-resistant *Staphylococcus aureus*, can be carried by them offering further opportunity for application. CYP51 has also emerged as a potential target for agents to treat *Leishmania* and trypanosome parasites by inhibiting ergosterol biosynthesis. Structures of these CYP51s have emerged to assist with new drug development for Chagas disease and sleeping sickness [167,168]. However, CYP5122A1 in *Leishmania donovani* has recently emerged as a novel potential drug target as it was found to be essential for survival [169]. Another major area where CYPs may be targets for new drugs is with mycobacteria where the target is not CYP51 [12,20], but potentially other essential features for growth or pathogenicity such as cholesterol catabolism by *Mycobacterium tuberculosis* CYP125 and CYP142 [170,171]. In *Mycobacterium leprae*, a massive decay of CYP and other genes has occurred through evolution with partial gene sequences still evident. The retention of CYP164A1 suggests this could be important and another drug target [172].

### 8. Concluding comments

The snapshot of microbial CYPs here reveals a fascinating diversity of functions and activities that have and will be of great use to humanity and the natural world. They have evolved specialized roles in helping organisms to compete in their niche. CYPs also promise to be a key to biotechnological adaptation to a post-oil-based economy producing chemical platforms for fuels, plastics and other products and combating global warming. In developing control agents for pathogens CYP-inhibitors help with human disease and increased food production. Better, more selective agents that degrade quickly after use will be important to reduce environmental effects and toxicities. New catalysts
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