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Exploiting cyanobacterial P450 pathways

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Cytochrome P450s are hemoprotein oxygenases involved in natural product synthetic pathways. Cyanobacteria are oxygenic photosynthetic microorganisms and are considered a rich source of natural products, and are now known to harbour P450s. A variety of cyanobacterial species have been found to contain multiple copies of P450s in their genomes, and over 100 have been predicted. Interestingly, some are membrane-bound as in eukaryotes, as opposed to cytoplasmic in bacteria. Furthermore, they can complement plant P450s and perform bioremediation of oil spills by the breakdown of alkanes. Functional expression of a selection *Nostoc* spp. P450s in *Escherichia coli*, with associated enzymes, has successfully produced the sesquiterpenes — germacradienol, germacrene and B-elemene, although others have failed for undetermined reasons.

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Introduction

Natural products have found use in health and disease for thousands of years, and they still form a major cornerstone of modern therapeutics [1,2]. These products fall into several therapeutic classes, including antineoplastic, antioxidant, antibiotic, cytotoxic, photo-protective, antiparasitic and antiviral [3•,4,5]. These natural products have not only served directly as drugs, but have also been used as primers for the discovery and synthesis of new drugs [1]. It was reported that between 1981 and 2006, 5% of the 1184 new chemical entities approved by the FDA were natural products and another 23% natural-product-derived molecules, with 30% as synthetic molecules [6]. According to the World Health Organisation (WHO), approximately 80% of the world population depends on traditional remedies for their primary health care needs [1]. These natural products are classified structurally into polyketides (PKS), nonribosomal peptides (NRPS), alkaloids, lipoproteins, phenylpropanoids and terpenes [4,7,8]. Most of these are secondary metabolites produced by organisms from conditional pathways that are activated in situations such as stress, developmental needs, signalling, starvation and defence [9]. These products are mainly sourced from plants and microbes, including ancient life-forms, cyanobacteria [10•].

Cyanobacteria are arguably one of the oldest known organisms on Earth, and are found in diverse habitats ranging from the oceans to fresh water bodies and to desert rocks [11]. These microbes are the only group of organisms that are able to reduce nitrogen and carbon in aerobic conditions, and thus play a significant role in the nitrogen and carbon cycles. They also play a major role in the evolution of life, by contributing to the oxygenation of the Earth's atmosphere [12]. Cyanobacteria are a rich source of natural products comprising both primary and secondary metabolites including nonribosomal proteins, polyketides and terpenes and alkaloids, and several of these are known to have anticancer, antiviral, UV protective activities as well as hepatotoxicity and neurotoxicity [12]. For example, the protein cyanovirin-N, derived from *Nostoc ellipsosporum*, has been employed to neutralise influenza A (H1N1) [13].

Many of the cyanobacterial bioactive compounds, such as the antitumor compound GSV224 from *Nostoc* sp., have been characterised and are known to be produced via the NRPS/PKS route [14,15]. However, several other non-NRPS/PKS synthetic pathways leading to the production of secondary metabolites with the active participation of cytochrome P450 (CYP) are just beginning to be appreciated, with the discovery of many CYP coding genes in their genome [16,17••]. Therefore, this review focuses on recent developments in P450 pathway research in the cyanobacteria.

Cytochrome P450s

The CYPs are a group of ubiquitous hemoprotein oxygenases that are found in all domains of life [18]. Over 11 000 genes encoding CYPs have been identified and are distributed in approximately 977 families to date. These are collected and updated by David Nelson in Memphis Tennessee (http://drnelson.utmem.edu/P450.statistics.Aug2009.pdf). The great diversity, occurrence and distribution of CYP suggests that they could be involved in essential or crucial metabolism such as defence against environmental pollutants, drugs detoxification, synthesis of important molecules [19,20]. CYPs are characterised by

their vivid orange–yellow pigment on complexation with carbon monoxide and form an intense Soret absorption maximum band at 450 nm in the UV spectrum (Table 1).

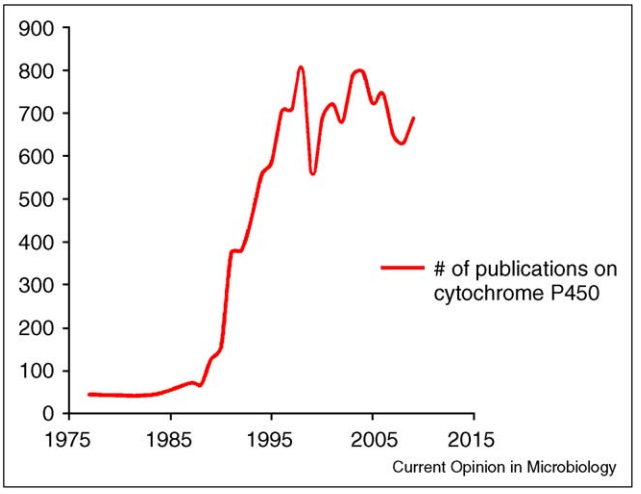
The CYP superfamily nomenclature is based on sequence similarity [56], and they are also classified on the basis of electron carrier protein type and system of electron delivery [21]. Characterisation of the crystal structures of several CYPs has given sufficient evidence of the unique fold, yet to be seen in other enzyme systems [22]. Despite less than 15% protein sequence homology across families, the structural core remains essentially conserved, suggesting that the tertiary structure evolved to bring all the essential components together irrespective of source or location [23].

The CYPs are involved in three fundamental functional roles in organisms. Firstly, they are needed for the synthesis of essential primary metabolites such as synthesis of steroid hormones in mammals required for growth and development and homeostatic regulation [24]. Secondly, they are responsible for metabolism and detoxification of exogenous molecules. These can then be removed from the organism as a protective measure, for example, the phase I biotransformation of drugs in humans by liver CYP [25]. Lastly, they are major players in the biosynthesis of many secondary metabolites, whose role to the organisms may not yet be evident, but which are highly sought for their useful roles as medicines [22,26••].

The classes of secondary metabolites produced by plants and microbes that have significant CYP activity include isoprenoids, alkaloids, phenylpropanoids and fatty acids as well as in the polyketide synthetic pathway [27,28]. Examples of useful products from secondary metabolic pathways catalysed by CYPs, include codeine, morphine and erythromycin [29–31].

Since the first paper on CYP was published in 1962, the volume of knowledge and publications has increased dramatically (Figure 1), with more than a thousand papers per annum since the late 1980s [32].

Figure 1



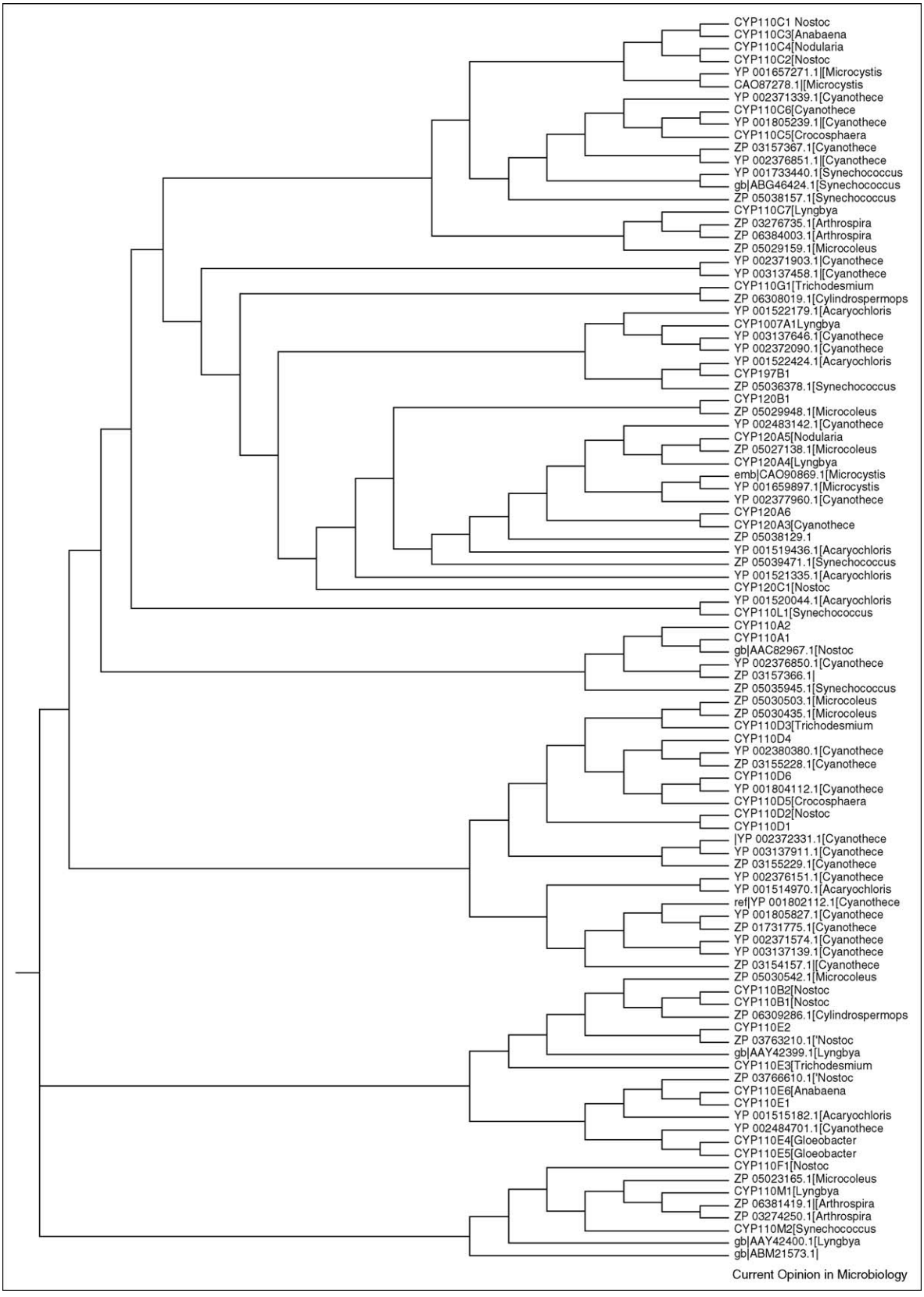
Number of cytochrome P450 publications since the 1970s. A survey of publications containing 'cytochrome P450' in their title, in the Web of Knowledge database (accessed: 18/01/2010) reveals that a least five hundred publications per annum has been maintained since the mid 1990s.

Cyanobacterial P450s

Several CYP and CYP-like genes have been identified in cyanobacterial genomes, however little attention has been given to their functional characterisation [33••,34••,35••]. The phylogenetic tree in Figure 2 shows the number of CYP containing cyanobacteria known-to-date. The fact that cyanobacteria, particularly those from the marine environment, are prolific producers of natural products [4,36,37,12,14,15], and with well-documented evidence of CYPs secondary metabolite production in other organisms, interest in cyanobacterial CYPs has amplified. There is evidence demonstrating that CYPs in cyanobacteria participate in specific metabolic processes, and are not the result of isolated gene transfer [33••,34••,35••]. For example, elucidation of the crystal structure of CYP120A1 has shown that it participates in retinoid metabolism [33••,34••]. Most natural products are formed in cyanobacteria through the NRPS, PKS or NRPS/PKS pathway [38,3•]. However, the first cloning, heterologous expression and functional characterisation of a CYP in a cyanobacterial species was conducted in 2005 [33••]. It is emerging that cyanobacteria have several CYPs in their genomes. A BLASTp search in the NCBI cyanobacterial genome database using the characterised protein NP_488726 from *Nostoc* sp. strain PCC 7120 [17••] or CYP120A1 from *Synechocystis* sp. PCC 6803 [34••] yields 100 putative CYP sequences distributed in most of the known cyanobacterial species (Figure 2). Sixty-three of these proteins have been characterised and named on the Cytochrome P450 Homepage (http://drnelson.utmem.edu/P450.statistics.Aug2009.pdf), with almost half belonging to the CYP110 family. Twelve fall into the CYP213 family, while the CYP120 family currently has 10 members. CYP110 from *Nostoc* sp. PCC 7120, unlike most bacterial CYPs, is not a cytosolic enzyme but membrane bound, like eukaryotic CYPs. It is not induced by alkanes, and does not participate in alkane biodegradation, but it is involved in ω-hydroxylation of long chain fatty acids and plays a role in nitrogen fixation [39].

Isoprenoids are one of the major structural classes of natural products. They are reputed to be the most diverse structurally, with an estimated 30 000–50 000 compounds already discovered to date [40]. They are

Figure 2



Phylogenetic tree of cytochrome P450 in cyanobacteria generated through BLASTp search in NCBI cyanobacterial database using NP_488726.

Table 1

Examples of cytochrome P450 catalysed bioactive natural products

Source organism	Compound	Activity	References
Lyngbya majuscula	Hectochlorin	Antifungal	[48]
Saccharopolypspora erythraea	Erythromycin	Antibacterial	[29]
Streptomyces nodosus	Amphotericin	Antifungal	[49]
Streptomyces avermitilis	Avermectin	Antiparasitic	[50]
Taxus brevifola	Taxol	Anticancer	[51]
Artemisia annua	Artemisinin	Antiparasitic/antimalarial	[52]
Papaver somniferum	Morphine	Opiate, analgesic	[53]
Catharanthus rosea	Vincristine	Anticancer	[54]
Aspergillus fumigatus	Fumitremorgin C	Anticancer	[55]

known to have a wide range of biological functions [41•]. Isoprenoid synthetic pathway research has been conducted mostly in plants and fungi [41•], but recent efforts have shown that cyanobacterial strains are capable of producing isoprenoids [17••]. In addition, marine cyanobacteria from *Microcoleus* and *Phormidium* genera have been known to contribute to bioremediation of oil spills using CYP as a catalyst for the alkane breakdown [42,43]. Agger *et al.* [17••] functionally expressed sesquiterpene synthase and CYP sourced from *Nostoc* sp. strain PCC 7120 and *Nostoc punctiforme* PCC 73102, in *Escherichia coli*, with limited success (which is the first known attempt). An oxidation product of the sesquiterpene synthase gene product was observed in *Nostoc* sp. strain PCC 7120 when co-expressed with CYP from the same strain, in *E. coli*. However, co-expression of sesquiterpene synthase from *N. punctiforme* PCC 73102 and the CYP from the same source yielded no oxidation product. Unfortunately, a second sesquiterpene synthase from *N. punctiforme* PCC 73102, which is a fusion-type protein, could not be overexpressed for *in vitro* characterisation. However, when transformed into *E. coli*, cultures were shown to produce the products, sesquiterpene (*E*,*E*)-germacradienol, germacrene and B-elemene. The sesquiterpene synthases from *Nostoc* sp. strain PCC 7120 and *N. punctiforme* PCC 73102 were functionally expressed, purified and shown to catalyse the *in vitro* cyclisation of farnesyl diphosphate to germacrene and 8a epi-α-selinene, respectively. Their failure to conclusively demonstrate the expression of the fusion-type sesquiterpene synthase and both CYPs as well as not showing the effect of CYP on the product of the sesquiterpene gene product may be due to a variety of reasons. Firstly, the wrong CYP from *N. punctiforme* PCC 73102 may have been cloned for the terpene synthase, as it now known that *N. punctiforme* PCC 73102 has at least 10 named CYP genes. Additionally, other regulatory genes may play a part in the pathway, as already speculated for the two-component proteins [44]. Secondly, expression of multiple plasmids and codon bias may have played a role, even though a pRARE plasmid was co-expressed. Functional expression of the CYP and *in vitro* characterisation is needed, as well as its reduction partner to confirm its functional role as was done for CYP120A1 in *Saccharomyces cerevisiae* [35••].

Conclusion and perspectives

CYPs are a superfamily of heme-thiolate proteins harbouring unique properties and found in all branches in the tree of life. They play crucial roles in a variety of important natural product biosynthetic pathways, including the production of commonly used drugs codeine and morphine. Cyanobacteria have been employed therapeutically before the advent of modern science, which has shown that they are prolific producers of natural products [45,4]. However, their importance as a source of natural product in CYP related pathways is only recently becoming apparent. The increasing number of functionally characterised CYPs from cyanobacteria, as well as evidence of terpene synthase genes, is opening new vista of natural product formation [17••,35••]. Cloning and functional characterisation of CYPs from cyanobacteria will have to be combined with elucidation of their electron transport partners, other regulatory genes and the pathways involved. For example, a systems biology based assessment of *Nostoc* sp. strain PCC 7120 and *N. punctiforme* PCC 73102 under native and conditional stress may be needed to evaluate terpene synthase and or CYP expression, and assist in identifying other proteins that may affect the pathway. More specifically, cloning and expressing the mevalonate pathway to provide and increase precursor flux for synthesis of terpene olefins in addition to using co-localised electron transport protein partners and codon optimisation may be required to improve results.

Ultimately, the need for cost effective and sustainable means of producing natural products in their natural form requires the application of metabolic engineering, systems and synthetic biology tools. This will not only involve pathway engineering, but also encourage structural engineering for more catalytically active CYP protein chimeras [46].

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