REVIEW

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Cytochromes P450 for natural product biosynthesis in *Streptomyces*: sequence, structure, and function

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Cytochrome P450 enzymes (P450s) are some of the most exquisite and versatile biocatalysts found in nature. In addition to their well-known roles in steroid biosynthesis and drug metabolism in humans, P450s are key players in natural product biosynthetic pathways. Natural products, the most chemically and structurally diverse small molecules known, require an extensive collection of P450s to accept and functionalize their unique scaffolds. In this review, we survey the current catalytic landscape of P450s within the *Streptomyces* genus, one of the most prolific producers of natural products, and comprehensively summarize the functionally characterized P450s from *Streptomyces*. A sequence similarity network of >8500 P450s revealed insights into the sequence–function relationships of these oxygen-dependent metalloenzymes. Although only ∼2.4% and <0.4% of streptomycete P450s have been functionally and structurally characterized, respectively, the study of streptomycete P450s involved in the biosynthesis of natural products has revealed their diverse roles in nature, expanded their catalytic repertoire, created structural and mechanistic paradigms, and exposed their potential for biomedical and biotechnological applications. Continued study of these remarkable enzymes will undoubtedly expose their true complement of chemical and biological capabilities.

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1   Cytochromes P450

Cytochromes P450 (P450s or CYPs) form a superfamily of ubiquitous heme-dependent enzymes that catalyze a diverse array of reactions *via* a complex multistep mechanism. They are most well known for their roles in xenobiotic detoxification, steroid biosynthesis, and drug metabolism in humans,[1,2] but also play key roles in the biosynthesis of natural products.[3–5] The heme (iron protoporphyrin IX) prosthetic group, which is coordinated on the proximal side by a thiolate ion, plays a key role in the reductive activation of molecular oxygen, a defining feature of P450 enzymes. P450s (P = pigment) were named as such due to their distinctive spectroscopic absorption maximum at 450 nm when the thiolate–ferrous–CO complex is reduced.[6,7] P450s are also commonly known as mono-oxygenases, due to the insertion of only one of the oxygen atoms from molecular oxygen into the substrate; the other is reduced to water. Correspondingly, hydroxylation of a carbon atom on a hydrophobic organic substrate is considered the archetypal P450 reaction. However, labeling a P450 as "just another hydroxylase" is a gross misrepresentation of their catalytic capabilities. The diversity and versatility of these natural bio-catalysts highlights nature's ability to evolve enzymes for chemical reactions.

P450s are of special interest to a variety of scientific disciplines. These oxygen-dependent metalloenzymes, with their fascinating chemical and physical properties, ability to catalyze C–H activation reactions, and diverse roles in human health, are relevant to pharmacologists and medicinal chemists,

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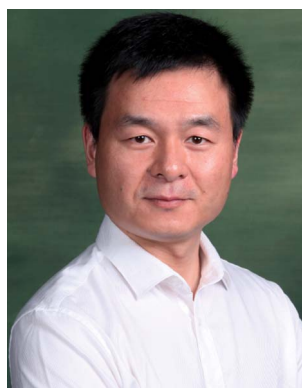
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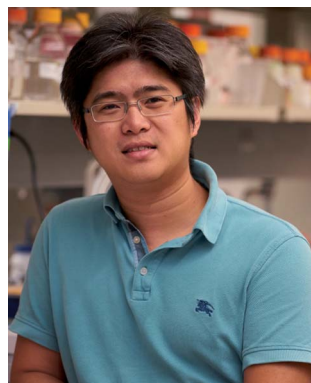
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enzymologists and biochemists, bioinorganic chemists and biophysicists, natural product chemists, and biomimetic and synthetic chemists. Consequently, P450s have been thoroughly reviewed from a variety of perspectives.[3,8–19] In this review, we aim to highlight the sequences, structures, and functions of P450s involved in the natural product biosynthetic pathways found in *Streptomyces* and how the study of these microbial enzymes has advanced the field of cytochrome P450 chemistry and enzymology.

2 Function

This review is not intended to extensively discuss the catalytic mechanism of P450s; it has been excellently covered elsewhere.[9,12,17,20–22] However, a description of the sophisticated P450 catalytic cycle is needed to discuss the ability of P450s to catalyze diverse chemical transformations.

The currently accepted P450 catalytic cycle, shown here as resulting in monooxygenation, is depicted in Fig. 1. In the resting state of the low-spin ferric (Fe^{III}) enzyme, a water molecule coordinates to the heme iron as the sixth ligand. Upon substrate binding, the coordinated water molecule is displaced, resulting in a change of the ferric iron spin state to high-spin. The iron, with its more positive reductive potential, is first reduced to the ferrous (Fe^{II}) state, molecular oxygen then binds, followed by a second reduction event resulting in the formation of a peroxo-ferric (Fe^{III}–OO^{2-}) intermediate. Two successive protonations of the distal oxygen, the first yielding the hydroperoxo-ferric intermediate Compound 0 (Cpd 0, Fe^{III}–OOH^{-}) and the second generating a transient intermediate (Fe^{III}–OOH_2), is followed by heterolytic fission of the O–O bond to yield the high valent oxo-ferryl (Fe^{IV}=O) π-cation porphyrin radical generally referred to as Compound I (Cpd I), while concomitantly releasing a water molecule. Cpd I then abstracts a hydrogen from the substrate forming a substrate radical species, which then, in the prototypical hydroxylation reaction, rebounds with the hydroxyl radical to form the hydroxylated product. Disassociation of the product from the enzyme allows water to return as the sixth heme ligand and finalizes the regeneration of the resting state of the hemoprotein. While Cpd I is accepted as the primary active species in heme oxygenase chemistry,[9] other intermediates are also capable of catalyzing oxidative reactions.[20,22,23] It should also be mentioned that several of the steps are likely in equilibrium and the rate-limiting step may vary, supporting a more dynamic view of the P450 catalytic cycle.[24]

The electrons required for heme-Fe^{III} reduction are generally supplied by NAD(P)H through redox protein partners.[10,25] There are two main classes of P450 redox systems: the bacterial and mitochondrial soluble class I and the eukaryotic microsomal membrane-bound class II. Typically, class I consists of a three-component system in which an NAD(P)H-dependent and FAD-containing reductase shuttles electrons to a [2Fe-2S] ferredoxin, which in turn shuttles the electrons to the P450 protein. The membrane-bound class II system is generally a two-component system that utilizes an NAD(P)H-dependent FAD- and FMN-containing reductase for electron supply. While most redox systems fit into either class I or II, there is a growing trend of exceptions, indicating there is still much to be uncovered regarding the diversity of P450 redox partners.[10] The most well-known and biotechnological useful examples include the fused multiprotein single-component systems P450$_{BM3}$ from *Bacillus megaterium*[26,27] and P450$_{RhF}$ from *Rhodococcus* sp.[28] The recent discoveries of the self-sufficient P450 CYP102D1 from *Streptomyces avermitilis*[29] and the redox-independent P450 CYP154A1 from *Streptomyces coelicolor*[30] continue to amend the paradigms of electron transfer systems for P450s.

One significant challenge of the bacterial three-component systems is to identify the native ferredoxin reductase and ferredoxin partners for each P450, and much work has focused on understanding these relationships in *Streptomyces*.[31–35] One advantage of bacterial P450s, and those from *Streptomyces* in particular, is the inherent flexibility of the P450s to accept electrons from heterologous redox partners. While each P450 behaves differently, streptomycete P450s have been shown to accept redox proteins from other *Streptomyces* species,[36–38] other bacterial genera including putidaredoxin reductase and putidaredoxin from the P450$_{cam}$ system in *Pseudomonas putida*[39,40] and flavodoxin reductase and flavodoxin from *Escherichia coli*,[41,42] and eukaryotes such as the commercially available spinach ferredoxin reductase and ferredoxin.[43,44] Inspired by the one-component systems, *Streptomyces* P450s have also been engineered into redox self-sufficient enzymes by the fusion of the P450 protein to the reductase domains of P450$_{RhF}$[45–47] or CYP102D1.[48] Finally, some P450s, are able to utilize H_2O_2 as a surrogate for the oxygens, electrons, and proton needed to directly generate Cpd 0 from the substrate-bound high-spin ferric P450 (*i.e.*, peroxide shunt or peroxygenation; Fig. 1).[9,49,50]
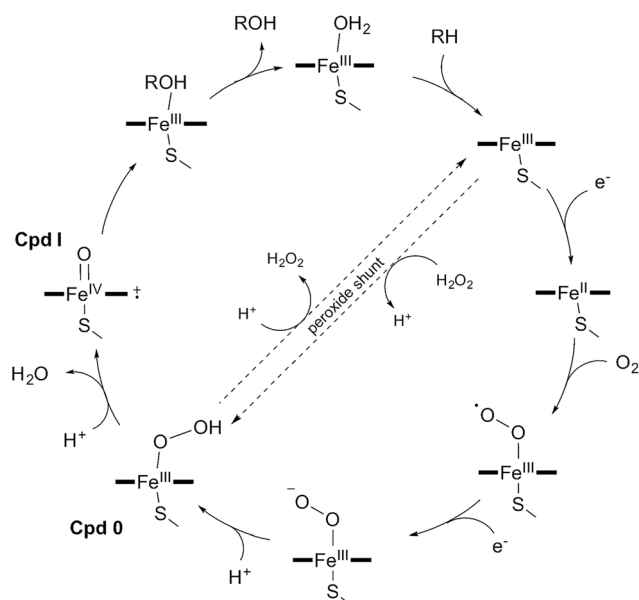

Fig. 1 The cytochrome P450 catalytic cycle. The cycle, shown here depicting hydroxylation of the substrate RH to yield the product ROH, is described in the text. The peroxide shunt pathway can directly form Cpd 0 from the substrate–bound high-spin Fe^{III} state using H_2O_2.

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2.1 P450s in natural product biosynthesis

Natural products are the most chemically and structurally diverse class of small molecules and consequently possess an extraordinarily wide range of biological activities.[51] The complexity of natural products arises from nature's ability to take simple building blocks and form complex and highly functionalized scaffolds. There are many strategies to biosynthesize natural products, but one common theme is the initial construction of a chemical scaffold followed by a set of reactions to functionalize the (typically) inert skeleton. Whether it be a linear or macrocyclic polyketide or a polycyclic terpenoid carbon skeleton, enzymes that can functionalize unactivated C–H bonds are vital to the production of biologically active natural products. The newly introduced functional groups can act as polar handles for substrate recognition, be used as attachment points for other moieties, or provide the chemical properties necessary for its mode of action or target interaction. Derivatization of the building blocks that nature uses adds another layer of complexity to the biosynthesis of natural products.

P450s are one of the most utilized enzymes that functionalize natural product scaffolds. This becomes anecdotally apparent when one considers two facts: (i) the sheer numbers of P450s found in the most prevalent producers of natural products, including plants, fungi, and the actinomycetes (see online databases http://drnelson.uthsc.edu/cytochromeP450.html and http://p450.riceblast.snu.ac.kr),[4,5,31,52,53] and (ii) the frequency of P450-encoding genes found within known and putative secondary metabolite gene clusters. The tremendous diversity in natural product structures requires diversity in the enzymes that act on them, and P450s are no exception. P450s that act in natural product biosynthetic pathways have some of the most diverse sequences, structures, functions, and mechanisms. It is because of these characteristics that natural product-related P450s have been crucial in revealing their diverse roles in nature, expanding their catalytic repertoire, creating structural and mechanistic paradigms, and exposing their potential for biomedical and biotechnological applications.

2.2 P450s in *Streptomyces*

After the genomes of *S. avermitilis*, the avermectin industrial strain, and *S. coelicolor*, the actinorhodin-producing model strain, were sequenced,[54–56] it was clear that the *Streptomyces* genus harbored a significant number of P450s. *S. coelicolor* and *S. avermitilis* contained 18 and 33 P450s, respectively, accounting for 0.2% and 0.4% of all coding sequences.[31,53] In comparison, many prokaryotes have only a few P450s encoded within their genomes; *Escherichia coli* and *Salmonella typhimurium* have none.[8,57] Since P450s are usually associated with either secondary metabolism or xenobiotic transformations, the number of P450s in *Streptomyces* and their diversity may reflect the extraordinary biosynthetic potential of these strains to produce diverse natural products, their ability to detoxify chemicals they come into contact with, or both.

Actinomycetes are generally considered as the workhorse producers of natural products, with *Streptomyces* being particularly proficient.[58] The recent emphasis on bacterial sequencing projects has continued to support these ideas, and it is now believed that we may have missed ∼90% of the natural product biosynthetic potential of actinomycetes.[58] These sequencing efforts and the subsequent explosion of new sequence data have also assembled an enormous library of orphan enzymes, *i.e.*, enzymes with unknown functions and/or unknown endogenous substrates.[11]

This review is limited to P450s from the *Streptomyces* genus for several reasons. Streptomycetes are one of the greatest reservoirs of natural products and the enzymes that catalyse their biosynthesis. Natural variants of enzymes found within natural product biosynthetic pathways have specialized functions that have evolved over millions of years to be catalytically efficient with regio- and stereoselectivities, and secondary metabolism-based P450s possess immense capacity for diverse chemical reactions. Streptomycetes are also traditionally more amenable to genetic manipulations, which allows the elucidation of the physiological functions of enzymes on endogenous substrates. The clustering of biosynthetic-related genes into operons or clusters, a common paradigm in microbial genomes,[59] also facilitates the identification of the endogenous natural product the P450 is involved in its biosynthesis. For these reasons, we considered *Streptomyces* to be an appropriate starting point to discuss the sequence–structure–function aspects of P450s involved in natural product biosyntheses.

There are 184 functionally characterized P450s of streptomycete origin (Table 1, access to the comprehensive P450 Excel spreadsheet is available online at http://www.scripps.edu/shen/NPLI/database.html). P450s were regarded as "functionally characterized" in this review if they met one or more of the following conditions: (i) the P450-encoding gene was cloned, the P450 protein was produced, and an *in vitro* experiment was conducted, or alternatively the protein was heterologously produced for biotransformation reactions (*i.e.*, *in vitro* characterization); (ii) the P450-encoding gene was inactivated in the native *Streptomyces* strain or was transferred to another host for genetic complementation (*i.e.*, *in vivo* characterization); or (iii) a crystal structure was solved (*i.e.*, structural characterization). Although there are many additional genes that encode P450s found in genomes and gene clusters or published in annotated gene cluster tables, mere functional prediction, comparison with highly homologous P450s, or having been named with the systematic nomenclature was not sufficient for inclusion in this "functionally characterized" collection. These sequences, however, were included in the P450 sequence similarity network (SSN), as discussed below.

2.3 Diverse functions

P450s are commonly associated with the hydroxylation, epoxidation, and dealkylation of xenobiotics found in human drug metabolism.[1] Yet, P450s are extremely versatile and their unique catalytic cycle plays a key role in bestowing these hemoproteins with a wide variety of functional capabilities.[16,21,23,24] These enzymes, although generally considered to show substrate promiscuity, can also be substrate specific,

Table 1 Experimentally characterized P450s of streptomycete origin

P450[a]	CYP name[b]	Biosynthetic pathway	Function(s)	In vitro[c]/in vivo/structure	PDB ID(s)	Ref.
AcmG8	—	Actinomycin G	Hydroxylation (PCP-tethered)[d]	—/Y/—		172
AknT	—	Aclacinomycin A	Glycosyltransferase (GTase) activator	Y/—/—		202 and 203
AmphL	161A3	Amphotericin B	Hydroxylation	—/Y/—		204
AmphN	105H4	Amphotericin B	Hydroxylation/oxidation to acid	—/Y/—		178 and 179
AryC	—	Arylomycin	Biaryl ring coupling (C–C)	—/Y/—		205
AurH	151A[e]	Aureothin	Hydroxylation/ether formation	Y/Y/Y	3P3L, 3P3O, 3P3X, 3P3Z	115–118
AveE	171A1	Avermectin	Ether formation	—/Y/—		119
AziB1	—	Azinomycin B	Hydroxylation	Y/—/—		74
BecO	1045A3	BE-14106	Hydroxylation	—/Y/—		206
BorI	—	Borrelidin	Hydroxylation/oxidation to aldehyde/oxidation to nitrile	Y/Y/—		90
BoxA	105A[e]	Xenobiotics	Hydroxylation	Y/—/—		131 and 207
CanC[f]	105H5	Candicidin	Hydroxylation	—/Y/—		208–211
ChoP	105C1	Unknown	Unknown	Y/—/—		212
ChryOIII	—	Chrysomycin	Desaturation	—/Y/—		213
CldC	—	Cyslabdan A	Hydroxylation/epoxidation	—/Y/—		214
ComI	165E1	Complestatin	Biaryl ring coupling (C–C)	—/Y/—		165
ComJ	165B5	Complestatin	Biaryl ring coupling (C–O)	—/Y/—		165
CotB3	—	Cyclooctatin	Hydroxylation	—/Y/—		215
CotB4	—	Cyclooctatin	Hydroxylation	—/Y/—		215
CSP4	107P3	Xenobiotics	Dealkylation	Y/—/—		216
CYP102B1	102B1	Fatty acids	Hydroxylation/epoxidation	Y/Y/—		31 and 217
CYP102D1	102D1	Fatty acids	Hydroxylation	Y/—/—		29
CYP105D4	105D4	Xenobiotics	Hydroxylation	Y/—/—		131
CYP105D5	105D5	Xenobiotics	Hydroxylation	Y/Y/—		27, 31, 32, 34 and 131
CYP105D6	105D6	Filipin	Hydroxylation	Y/—/Y	3ABB	218
CYP105D7	105D7	Pentalenolactone/ xenobiotics	Hydroxylation	Y/Y/Y	4UBS	48 and 219–224
CYP105F2	105F2	Oleandomycin	Hydroxylation	Y/—/—		225
CYP105N1	105N1	Coelibactin	Unknown	Y/—/Y	3TYW, 4FXB	31, 226 and 227
CYP105P1	105P1	Filipin	Hydroxylation	Y/—/Y	3ABA, 3E5J, 3E5K, 3E5L	218 and 228
CYP105P2	105P2	Flavones	Hydroxylation	Y/—/Y	5IT1	40, 190 and 229
CYP107AJ1	107AJ1	Xenobiotics	Dealkylation	Y/—/—		154
CYP107L2	107L2	Fatty acids	Unknown	Y/—/Y	5CJE, 5CWE	230
CYP107P1	107P1	Unknown	Unknown	Y/—/—		31
CYP107P2	107P2	Xenobiotics	Hydroxylation	Y/—/—		171
CYP107T1	107T1	Unknown	Unknown	Y/—/—		31
CYP107U1	107U1	Xenobiotics	Oxidation to ketone	Y/Y/—		31 and 231
CYP107W1	107W1	Oligomycin A	Hydroxylation	Y/—/Y	4WPZ, 4WQ0	232 and 233
CYP107Y1	107Y1	Xenobiotics	Hydroxylation	Y/—/—		171
CYP107Z13	107Z13	Avermectin	Oxidation to ketone	Y/—/—		133 and 234
CYP125A2	125A2	Xenobiotics	Hydroxylation	Y/—/—		171
CYP147F1	147F1	Fatty acids	Hydroxylation	Y/—/—		235–237
CYP154A1	154A1	Dipentaenone/ xenobiotics	Cycloaddition/dealkylation	Y/Y/Y	1ODO	30–32, 188, 207 and 238
CYP154C1	154C1	Pikromycin/ methymycin/ neomethymycin	Hydroxylation	Y/—/Y	1GWI	31, 32 and 239
CYP154C3	154C3	Xenobiotics	Hydroxylation	Y/—/—		45
CYP155A1	155A1	Unknown	Unknown	Y/—/—		31
CYP156A1	156A1	Unknown	Unknown	Y/—/—		31
CYP156B1	156B1	Unknown	Unknown	Y/—/—		31
CYP157A1	157A1	Unknown	Unknown	Y/—/—		31
CYP157B1	157B1	Unknown	Unknown	Y/—/—		31
CYP157C1	157C1	Unknown	Unknown	Y/—/—		31 and 151
CYP157C4	157C4	Xenobiotics	Dealkylation	Y/—/—		240
CYP158A1	158A1	Flaviolin	Biaryl ring coupling (C–C)	Y/—/Y	2DKK, 2NZ5, 2NZA	31, 32 and 42

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Table 1 (Contd.)

P450[a]	CYP name[b]	Biosynthetic pathway	Function(s)	In vitro[c]/in vivo/structure	PDB ID(s)	Ref.
CYP158A2	158A2	Flaviolin	Biaryl ring coupling (C–C)	Y/—/Y	1SE6, 1S1F, 1T93, 2D0E, 2D09, 3TZO, 5DE9	31, 32, 42, 102, 111 and 112
CYP159A1	159A1	Unknown	Unknown	Y/—/—		31
CYP170A1[f]	170A1	Albaflavenone	Hydroxylation/oxidation to ketone/diphosphate ionization	Y/Y/Y	3DBG, 3EL3	145–147 and 241
CYP170A2	170A2	Albaflavenone	Hydroxylation/oxidation to ketone	—/Y/—		242
CYP170B1	170B1	Albaflavenone	Hydroxylation/oxidation to ketone	Y/—/—		147
CYP450Y110	—	Xenobiotics	Hydroxylation	Y/—/—		243
CYP51[f]	170A1	Xenobiotics	Dealkylation	Y/Y/—		244
CYPSvh01[f]	105C1	Xenobiotics	Hydroxylation	Y/—/—		245
CYPSvu022	154H[e]	Xenobiotics	Hydroxylation	Y/—/—		245
DesVIII	—	Pikromycin	GTase activator	Y/Y/—		141–143, 246 and 247
DnrQ	131A1	Duanorubicin	GTase activator	—/Y/—		143, 246 and 248
DoxA27952	129A2	Doxorubicin	Hydroxylation/oxidation to ketone	Y/—/—		35
DoxA29050	129A2	Doxorubicin	Hydroxylation/oxidation to ketone	Y/Y/—		86, 249 and 250
DoxAC5	129A1	Doxorubicin	Hydroxylation/oxidation to ketone	Y/—/—		251 and 252
Ema1	107Z12	Xenobiotics	Hydroxylation/oxidation to ketone	Y/—/—		132
Ema2[f]	107Z10	Xenobiotics	Hydroxylation/oxidation to ketone	Y/—/—		132
Ema3	107Z2v2	Xenobiotics	Hydroxylation/oxidation to ketone	Y/—/—		132
Ema4[f]	107Z5v3	Xenobiotics	Hydroxylation/oxidation to ketone	Y/—/—		132
Ema5	107Z6	Xenobiotics	Hydroxylation/oxidation to ketone	Y/—/—		132
Ema6	107Z5v2	Xenobiotics	Hydroxylation/oxidation to ketone	Y/—/—		132
Ema7	107Z3	Xenobiotics	Hydroxylation/oxidation to ketone	Y/—/—		132
Ema8	107Z2v1	Xenobiotics	Hydroxylation/oxidation to ketone	Y/—/—		132
Ema9	107Z11	Xenobiotics	Hydroxylation/oxidation to ketone	Y/—/—		132
Ema10[f]	107Z5v3	Xenobiotics	Hydroxylation/oxidation to ketone	Y/—/—		132
Ema11	107Z1	Xenobiotics	Hydroxylation/oxidation to ketone	Y/—/—		132
Ema12	107Z9	Xenobiotics	Hydroxylation/oxidation to ketone	Y/—/—		132
Ema13	107Z8	Xenobiotics	Hydroxylation/oxidation to ketone	Y/—/—		132
Ema14[f]	107Z10	Xenobiotics	Hydroxylation/oxidation to ketone	Y/—/—		132
Ema15	107Z5v1	Xenobiotics	Hydroxylation/oxidation to ketone	Y/—/—		132
Ema16	107Z4	Xenobiotics	Hydroxylation/oxidation to ketone	Y/—/—		132
Ema17	107Z7	Xenobiotics	Hydroxylation/oxidation to ketone	Y/—/—		132
EncR	107R1	Enterocin	Hydroxylation	Y/Y/—		253 and 254
FcpC[f]	105[e]	Xenobiotics	Hydroxylation	Y/—/—		38
FilC	—	Filipin	Hydroxylation	—/Y/—		255
FilD	—	Filipin	Hydroxylation	—/Y/—		255
FosK	—	Fostriecin	Hydroxylation	—/Y/—		256
FscP[f]	105H5	Candicidin	Hydroxylation/oxidation to acid	—/Y/—		209–211
GalD	—	Galbonolide	Hydroxylation or epoxidation	—/Y/—		257
GbnD	—	Galbonolide	Hydroxylation or epoxidation	Y/—/—		258
GdmP	105U1	Geldanamycin	Desaturation	—/Y/—		92–94 and 259
GerPI	—	Dihydrochalcomycin	Epoxidation	—/Y/—		260
GerPII	—	Dihydrochalcomycin	Hydroxylation	—/Y/—		260
GfsF	105[e]	FD-891	Hydroxylation/epoxidation	Y/Y/—		261 and 262
GilOIII	—	Gilvocarcin	Desaturation	—/Y/—		213 and 263
GrhO3	105D9	Griseorhodin	Epoxidation	—/Y/—		264
HerG	—	Herboxidiene	Hydroxylation	Y/—/—		265 and 266
HerO	—	Heronamide	Hydroxylation	Y/Y/—		46
HlsH	107[e]	Halstoctacoanolide	Hydroxylation/oxidation to ketone	—/Y/—		164
HlsI	107[e]	Halstoctacoanolide	Hydroxylation	—/Y/—		164
HmtN	—	Himastatin	Hydroxylation	Y/Y/Y	4E2P	95 and 126
HmtS	—	Himastatin	Biaryl ring coupling (C–C)	—/Y/—		95
HmtT	—	Himastatin	C–N bond formation	Y/Y/Y	4GGV	95 and 126
JulI	—	Julichrome	Biaryl ring coupling (C–C)	Y/Y/—		96
LkmF	107A2	Lankomycin	Hydroxylation	—/Y/—		267 and 268
LkmK	107AP1	Lankomycin	Hydroxylation	—/Y/—		267 and 268
LnmA	107AC1	Leinamycin	Hydroxylation	Y/Y/Y	4Z5P	269
LnmZ	107AG1	Leinamycin	Hydroxylation	Y/Y/Y	4Z5Q	269
LtmK	—	Lactimidomycin	Desaturation	—/Y/—		91

Table 1 (Contd.)

P450[a]	CYP name[b]	Biosynthetic pathway	Function(s)	In vitro[c]/in vivo/structure	PDB ID(s)	Ref.
MeiE	171A2	Meilingmycin	Ether formation	—/Y/—		173 and 174
MfnN	—	Marfomycin	Hydroxylation	—/Y/—		270
MgsK	—	iso-Migrastatin	Hydroxylation	—/Y/—		271
MonD	124B1	Monensin	Hydroxylation (ACP-tethered)[d]	—/Y/—		79
NcsB3	154J1	Neocarzinostatin	Hydroxylation	Y/—/—		272 and 273
NikF	105K1	Nikkomycin	Hydroxylation	—/Y/—		167 and 274
NikQ	162A1	Nikkomycin	Hydroxylation (PCP-tethered)	Y/Y/—		167 and 168
NovI	163A1	Novobiocin	Hydroxylation (PCP-tethered)[d]	Y/—/—		166
NysL	161A1	Nystatin	Hydroxylation	Y/Y/—		180, 181 and 275
NysN	105H1	Nystatin	Hydroxylation/oxidation to acid	—/Y/—		180 and 181
NzsA	—	Neocarazostatin	Hydroxylation	Y/Y/—		276
OleP	107D1	Oleandomycin/ xenobiotics	Epoxidation/hydroxylation	Y/—/Y	4XE3	72, 73, 131, 277 and 278
OleP1	235A1	Oleandomycin	GTase activator	—/Y/—		248 and 279
ORF-A	107C1	Carbomycin	Epoxidation	Y/—/—		280
P450$_{CLA}$	105M1	Clavulanic acid	Unknown	Y/—/—		281
P450$_{mel}$	107F1	Melanin	Biaryl ring coupling (C–C)	Y/Y/—		103
P450$_{sca-2}$	105A3	Xenobiotics	Hydroxylation	Y/—/—		43 and 282–285
P450$_{sky}$	163B3	Skyllamycin	Hydroxylation (PCP-tethered)[d]	Y/Y/Y	4L0E, 4L0F, 4PWV, 4PXH	76–78
P450$_{SU-1}$	105A1	Xenobiotics	Hydroxylation/epoxidation/ dealkylation	Y/Y/Y	2ZBX, 2ZBY, 2ZBZ, 3CV8, 3CV9	36, 131, 134–137, 139 and 286–294
P450$_{SU-2}$	105B1	Xenobiotics	Hydroxylation/dealkylation	Y/Y/—		36, 138, 286–289 and 295
P450$_{terf}$	107L[e]	Xenobiotics	Hydroxylation	Y/Y/—		296 and 297
PenM	161C3	Pentalenolactone	Oxidative rearrangement	Y/Y/—		113
PikC	107L1	Pikromycin/ methymycin/ neomethymycin/ xenobiotics	Hydroxylation/oxidation to ketone	Y/Y/Y	2BVJ, 2CA0, 2CD8, 2C6H, 2C7X, 2VZM, 2VZ7, 2WH2, 2WI9, 3ZK5, 3ZPI, 4B7D, 4B7S, 4BF4, 4UMZ	47, 65–69 and 298–306
PimD	161A2	Pimaricin	Epoxidation	Y/Y/Y	2XBK, 2X9P	50, 70 and 71
PlaO2	—	Phenalinolactone	Nonfunctional	—/Y/—		83
PlaO3	—	Phenalinolactone	Hydroxylation	—/Y/—		82
PlaO4	—	Phenalinolactone	Hydroxylation	—/Y/—		83
PlaO5	—	Phenalinolactone	Hydroxylation	Y/Y/—		83
PldB	107[e]	Pladienolide	Hydroxylation	Y/Y/—		307
PlmS2	107L8	Phoslactomycin	Hydroxylation	Y/Y/—		308
PntM	161C2	Pentalenolactone	Oxidative rearrangement	Y/Y/Y	5L1O, 5L1P, 5L1Q, 5L1R, 5L1S, 5L1T, 5L1U, 5L1V, 5L1W	113 and 114
ProP450	—	Xenobiotics	Hydroxylation	Y/—/—		49 and 309
PsmA	105[e]	Pladienolide	Hydroxylation	Y/Y/—		310,311
PtlI	183A1	Pentalenolactone	Hydroxylation/oxidation to aldehyde	Y/—/—		312
PtmO5	—	Platensimycin	Ether formation	—/Y/—		120
Qui15	—	Echinomycin	Hydroxylation (PCP-tethered)[d]	—/Y/—		170
RapJ	122A2	Rapamycin	Hydroxylation	—/Y/—		313 and 314
RapN	107G1	Rapamycin	Hydroxylation	—/Y/—		313 and 314
RavOIII	—	Ravidomycin	Desaturation	—/Y/—		213
RevI	—	Reveromycin	Hydroxylation	Y/Y/Y	3WVS	315
RmnC	—	Raimonol	Hydroxylation	—/Y/—		214
SamR0478	—	Stambomycin	Hydroxylation	—/Y/—		316
SamR0479	—	Stambomycin	Hydroxylation	—/Y/—		316
SanH	105K2	Nikkomycin	Hydroxylation	—/Y/—		317 and 318
SanQ	162A2	Nikkomycin	Hydroxylation (PCP-tethered)	—/Y/—		169
Sclav_p0067	—	(−)-Drimenol	Unknown[g]	—/Y/—		319
ScnD	—	Pimaricin	Epoxidation	Y/Y/—		182 and 183
ScnG	—	Pimaricin	Hydroxylation/oxidation acid	—/Y/—		182 and 183
SgcD3	211A1	C-1027	Hydroxylation	—/Y/—		320
SgvP	107[e]	Griseoviridin	C–S bond formation	Y/Y/Y	4MM0	129, 130 and 189
SKCTCFkbD[f]	—	FK506/FK520	Hydroxylation/oxidation to ketone	Y/Y/—		321
Sl5NT	—	Unknown	Nitration	Y/—/—		125

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Table 1 (*Contd.*)

P450[a]	CYP name[b]	Biosynthetic pathway	Function(s)	*In vitro[c]/in vivo*/structure	PDB ID(s)	Ref.
SlgO2	—	Streptolydigin	Hydroxylation/oxidation to ketone	—/Y/—		322 and 323
SlStaN	244A1	Staurosporine	C–N bond formation	Y/—/—		127
SMAFkbD	—	FK506/FK520	Hydroxylation/oxidation to ketone	—/Y/—		324
SMg15NT	—	Unknown	Nitration	Y/—/—		125
SoCYP158A2	158A2	Phenol/indole	Hydroxylation	Y/—/—		325
SoyC	105D1	Xenobiotics	Hydroxylation/epoxidation/ dealkylation/desaturation	Y/—/—		37 and 326–333
Srm13	—	Spiramycin	Hydroxylation/oxidation to aldehyde	—/Y/—		334 and 335
StaF	165A4	A47934	Biaryl ring coupling (C–O) (PCP-tethered)	Y/Y/Y	5EX8, 5EX9	99, 106 and 107
StaG	165D1	A47934	Biaryl ring coupling (C–O)	—/Y/—		99
StaH	165B4	A47934	Biaryl ring coupling (C–O) (PCP-tethered)	Y/Y/Y	5EX6	99, 106 and 107
StaJ	165C5	A47934	Biaryl ring coupling (C–C)	—/Y/—		99
StaP	245A1	Staurosporine	Biaryl ring coupling (C–C)/ oxidative decarboxylation	Y/—/Y	2Z3T, 2Z3U, 3A1L	100, 101, 108–110 and 336
StFkbD[f]	—	FK506/FK520	Hydroxylation/oxidation to ketone	Y/Y/—		337 and 338
StStaN	244A1	Staurosporine	C–N bond formation	—/Y/—		100 and 128
StuD1	—	Thiolactomycin/ thiotetronate	Epoxidation (ACP-tethered)[d]	—/Y/—		80
StuD2	—	Thiolactomycin/ thiotetronate	Hydroxylation/oxidation to acid	—/Y/—		80
Sv5NT	—	Unknown	Nitration	Y/—/—		125
TamI[f]	—	Tirandamycin	Hydroxylation/oxidation to ketone/epoxidation	Y/Y/—		84 and 85
TetrK[f]	—	Tetramycin	Hydroxylation	Y/Y/—		339
ThnC	—	Thienodolin	C–S bond formation	Y/Y/—		340
TmcR	—	Tautomycetin	Hydroxylation/oxidation to ketone	—/Y/—		341 and 342
TrdI[f]	—	Tirandamycin	Hydroxylation/oxidation to ketone/epoxidation	—/Y/—		343
TtmD[f]	—	Tetramycin	Hydroxylation	Y/Y/—[g]		339 and 344
TtnI	—	Tautomycetin	Hydroxylation/oxidation to ketone	—/Y/—		345
TxtC	246A1	Thaxtomin	Hydroxylation	Y/Y/—		346
TxtE	—	Thaxtomin	Nitration	Y/Y/Y	4L36, 4TPO, 4TPN, 5D3U, 5D40	122–125 and 347
TylHI	105L1	Tylosin	Hydroxylation	—/Y/—		348–350
TylMIII	—	Tylosin	GTase activator	—/Y/—		143, 246 and 351
Unk. P450	—	Xenobiotics	Dealkylation	—/Y/—		352
XiaM	—	Xiamycin	Hydroxylation/oxidation to acid	Y/Y/—		89

[a] Commonly used P450 names. Duplicated names are differentiated by adding the first letter of the genus and species, *e.g.*, SlStaN = *Streptomyces longisporoflavus* StaN. [b] CYP names identified from reference or the Cytochrome P450 Homepage (http://drnelson.uthsc.edu).[163] [c] In this study, heterologous P450 biotransformations are considered *in vitro* experiments. [d] P450 acts on a PCP- or ACP-tethered substrate. [e] Only CYP family or subfamily was given. [f] CanC = FscP; CYP170A1 = CYP51; CYPSvh01 = FcpC; Ema2 = Ema14; Ema4 = Ema10; StFkbD = SKCTCFkbD; TamI = TrdI; TetrK = TtmD. [g] The function of Sclav_p0067 is still unknown, but *in vivo* results support alteration of FPP cyclization by the sesquiterpene synthase Sclav_p0068.[319]

showing regio- and stereoselectivity, particularly when involved in the biosynthesis of natural products. As described in Section 2, many P450 functions can be attributed to the use of Cpd I as the oxidizing species. It is thought that the structural diversity of natural products, translating into diverse electronic properties, may allow P450 catalytic intermediates that are less active than Cpd I to act as alternative oxidants.[3] This section aims at highlighting selected examples of *Streptomyces* P450 to give an overview of the types of P450 transformations that are known to occur on the structurally diverse skeletons of natural products (Fig. 2).

2.3.1 Oxygenation. Hydroxylation of an aliphatic carbon is the prototypical P450 transformation, but P450s can also oxygenate carbons by epoxidation and aromatic hydroxylation. When involved in the functionalization of natural products, these P450 oxidative reactions are very often stereo- and regio-selective, resulting in retention of configuration.[12] C–H activation, one of the most difficult and most sought after synthetic and biosynthetic reactions,[60–62] is a trademark of P450s. It is this ability, to insert an oxygen into an unactivated C–H bond, that piques the interest of synthetic and biosynthetic chemists, making P450s one of the most targeted types of enzymes for biotechnological applications.[13,14,19,63,64]
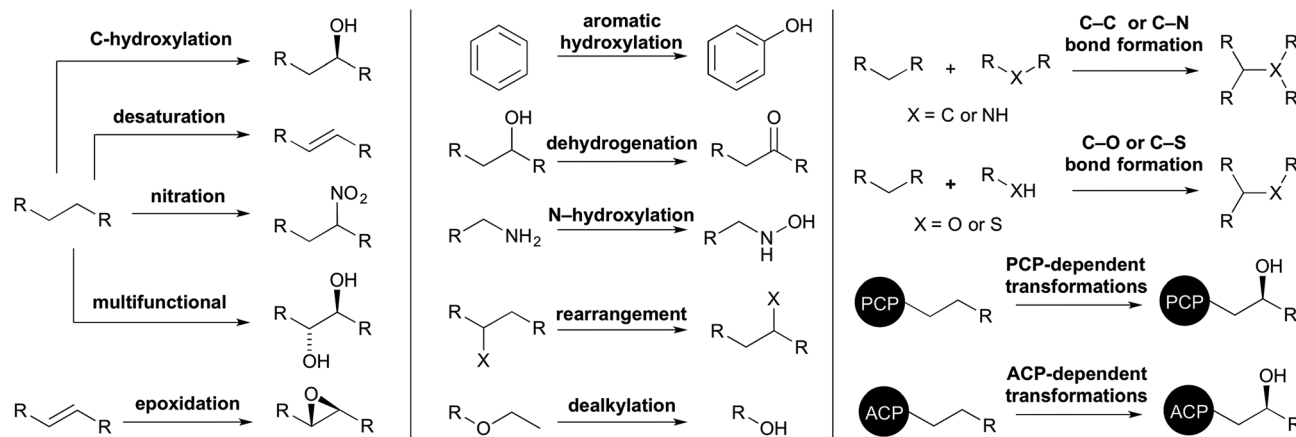
Fig. 2 The functional diversity of P450s in *Streptomyces*. P450s catalyze a wide variety of functionalizations (selected examples are shown) in natural product biosynthetic pathways and in xenobiotic degradation.

The majority of P450s in natural product biosynthetic pathways catalyze hydroxylation. While much of this review focuses on the sequence, structure, and function of P450s that catalyze other types of reactions, it should be noted that more than two-thirds of the characterized P450s from *Streptomyces* catalyze hydroxylations (Table 1). The most well known and studied P450 from *Streptomyces*, PikC (CYP107L1), catalyzes the aliphatic hydroxylation of several different macrolide antibiotics. PikC hydroxylates 12-membered macrolides to form methymycin and neomethymycin, and can hydroxylate twice at different positions to yield novamethymycin (Fig. 3).[65,66] PikC also hydroxylates the 14-membered macrolide narbomycin to give pikromycin.[67–69]

In addition to hydroxylation, P450s also commonly convert C–C double bonds into epoxides with retention of configuration.[22] P450 epoxidations are frequently seen on polyketides due to the prevalence of olefins. PimD (CYP161A2), which epoxidizes the C-4–C-5 conjugated olefin in the polyene macrolide pimaricin (Fig. 3), is likely catalyzed by Cpd 0 (discussed in more detail in Section 4.3.1).[50,70,71] OleP (CYP107D1), a P450 epoxidase responsible for the C-8–C-8a epoxide in oleandomycin (Fig. 3), is unique in that it catalyzes epoxidation of two aliphatic carbons, although there is evidence that this transformation proceeds through an OleP-generated olefinic intermediate.[72,73]

P450s can also hydroxylate aromatic carbons, with the hydroxylation of naphthoic acid by AziB1 in the biosynthesis of azinomycin B as one example from *Streptomyces* (Fig. 3).[74] The commonly accepted mechanism for P450 aromatic hydroxylation is initial epoxidation of the aromatic ring, followed by epoxide opening and rearomatization *via* hydride migration.[8,75]

Most P450s bind free substrates, but some achieve substrate selectivity by directly interacting with the peptidyl-carrier protein (PCP) of a nonribosomal peptide synthetase (NRPS) or the acyl-carrier protein (ACP) of a polyketide synthase (PKS), respectively. P450$_{sky}$ (CYP163B3) was demonstrated to utilize PCP-tethered amino acids as substrates in the modular biosynthesis of the cyclic depsipeptide skyllamycin.[76–78] P450$_{sky}$

β-hydroxylates L-Phe, OMe-L-Tyr, and L-Leu in modules 5, 7, and 11, respectively (Fig. 3), but does not hydroxylate the PCP-tethered amino acids in the other eight modules of the NRPS, indicating that P450$_{sky}$ harbors innate PCP domain selectivity.[77] P450s that utilize ACP-tethered substrates are also involved in natural product biosynthetic pathways, including monensin[79] and thiolactomycin,[80] although direct evidence is yet to be established. P450–ACP complexes, however, have been reported in other systems.[81]

A common theme throughout natural product biosynthesis is the late-stage functionalization at various positions on the scaffold. Many biosynthetic gene clusters contain multiple P450s to functionalize one scaffold, either sequentially or with additional modification steps in between each P450-catalyzed step. There are four P450-encoding genes, *plaO2–plaO5*, within the phenalinolactone gene cluster.[82] With the exception of PlaO2, each P450 catalyzes the hydroxylation of a different carbon on the diterpene scaffold. PlaO3 and PlaO4 hydroxylate the C-20 and C-21 methyl groups, respectively, and PlaO5 stereoselectively hydroxylates C-1 (Fig. 3).[82,83] PlaO2 was determined to be nonessential in phenalinolactone formation,[83] although it is not clear whether it is nonfunctional, can be complemented by another P450, or has another biological role in the organism. An alternative strategy to functionalize one scaffold multiple times is the utilization of a multifunctional P450, with TamI being the quintessential example. Acting through repeated exchange with the FAD-dependent oxidase TamL, TamI catalyzes an oxidative cascade on the tirandamycin skeleton, beginning with stereoselective hydroxylation at C-10, and followed by successive epoxidation at C-11–C-12 and hydroxylation of the C-18 methyl (Fig. 3).[84,85] TamI, in the absence of TamL, can also bypass the TamL dehydrogenation reaction and form minor amounts of the C-10 ketone.[85]

2.3.2   Dehydrogenation. P450s are also known to catalyze two or more electron dehydrogenations, converting an sp^3 hybridized carbon into an sp^2 or sp hybridization state. The classification of P450 "dehydrogenations" can sometimes be a misnomer, however, due to ketones, aldehydes, and carboxylic
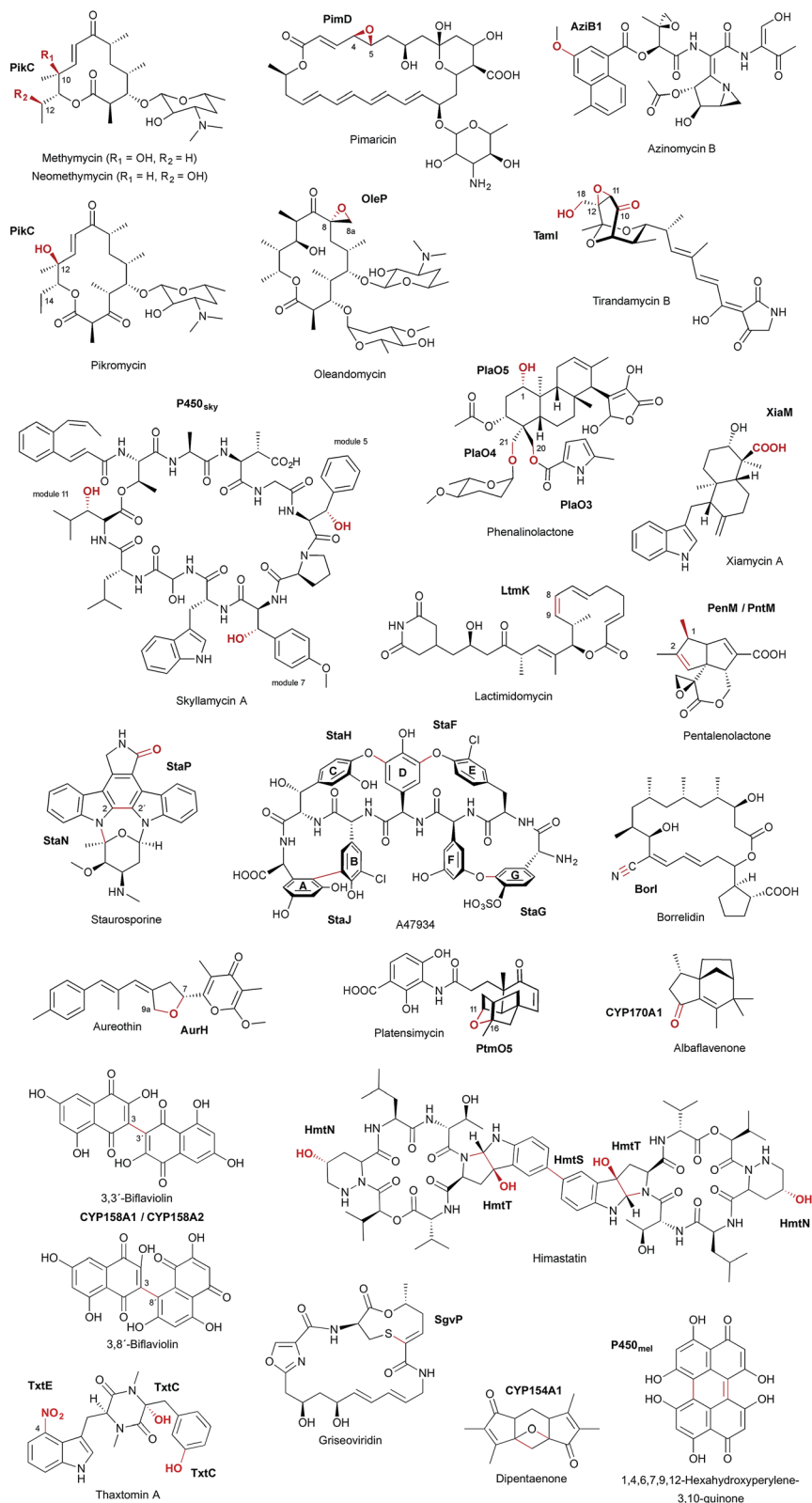
Fig. 3 Selected natural products, P450s, and the biosynthetic transformations they catalyze, as discussed in the text. Functional groups and bonds colored in red are catalyzed by the P450s labeled in bold.

acids forming through second or third hydroxylations with subsequent (likely) nonenzymatic dehydrations.[21,75,86–88] In Table 1, these "dehydrogenations" are simply labeled as "oxidations". For example, XiaM, which converts a methyl into a carboxylic acid in the biosynthesis of the indolosesquiterpene alkaloid xiamycin A biosynthesis (Fig. 3), was shown to have

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hydroxyl, geminol diol, and aldehyde intermediates.[89] Another extraordinary example is BorI.[90] BorI first produces an aldehyde group from a methyl in a four electron oxidative process. After the aldehyde undergoes BorJ-mediated transamination, BorI completes the conversion of the amine into a nitrile, presumably through *N,N*-dihydroxy and aldoxamine intermediates (Fig. 3).[90]

The other subset of dehydrogenation reactions are performed by the P450 desaturases. When Cpd I creates the substrate radical, there is a competition between hydrogen atom abstraction and hydroxylation. Most often, the hydroxylated compound is the major product, and the hydroxylated and desaturated congeners can not be interconverted.[21] In the biosynthesis of some natural products, desaturation is the predominant P450 product, supporting that desaturation can be enzymatically controlled. In the final step of lactimidomycin, LtmK converts 8,9-dihydro-LTM into LTM at >95% efficiency (Fig. 3).[91] The presence of <5% of both the 8*S*- and 9*R*-hydroxy-LTM analogues (hydroxyl groups are on the same face), suggests that LtmK can generate a radical on either the C-8 or C-9 position. GdmP (CYP105U1), a desaturase in geldanamycin biosynthesis,[92,93] may also allow radical rebound to the heme-bound oxygen in a non-stereoselective manner as both stereochemistries of the C-4 hydroxylated congener were isolated from the producing strain.[94]

2.3.3 Biaryl ring coupling. The coupling of aromatic rings can create structural diversity (*e.g.*, himastatin[95] and julichromes[96]), give flexible backbones rigid conformations [*e.g.*, glycopeptide antibiotics (GPA)[97–99] and staurosporine[100,101]], and polymerize monomers to yield natural sunscreens (*e.g.*, melanins[102,103]). The signature structural feature in the GPA family, the most well known being vancomycin, is the heptapeptide backbone with multiple biaryl (C–C) or biaryl ether (C–O–C) bridges.[104] Linear and incompletely processed intermediates or analogues do not retain the strong antibacterial activities associated with these natural products.[97] In *Streptomyces*, the GPA A47934 has four of these aromatic bridges;[105] the vancomycin-type only has three.[104] Four CYP165 members, StaH, StaG, StaF, and StaJ, sequentially install the C–O–D, F–O–G, D–O–E, and A–B rings, respectively (Fig. 3).[99,106] Adding to the complexity and interest of this family of P450s, they utilize PCP-tethered substrates and interact with the so-called X-domain, found in the last NRPS module, for P450 recruitment and cyclization efficiency.[97,98,107]

StaP (CYP245A1) is responsible for intramolecular C–C bond formation in staurosporine biosynthesis. StaP was proposed and theoretically supported to delocalize the π-cation porphyrin radical of Cpd I over the indole moieties of chromopyrrolic acid (CPA).[108,109] CPA, upon losing an electron and proton, forms a C–C bond between the two indole rings at C-2 with a coupled second electron transfer, and goes through a series of protonations and tautomerizations to yield the six-ring indolocarbazole (Fig. 3).[108,109] After biaryl coupling of CPA, StaP also catalyzes oxidative decarboxylation, mediated by the FAD-dependent enzyme StaC or RebC (from the rebeccamycin pathway).[101,110]

CYP158A1 and CYP158A2 from *S. coelicolor* polymerize flaviolins to form a variety of bi- and tri-flaviolins that may protect against UV radiation.[42,102,111,112] Although these two P450s share 61% sequence identity and bind the same substrate, CYP158A1 only forms two biflaviolins, whereas CYP158A2 forms at least four polymers (Fig. 3). Similarly, P450mel (CYP107F1) in *Streptomyces griseus* catalyzes sequential oxidations of 1,3,6,8-tetrahydroxynaphthalene (THN), first through an intermolecular aryl coupling and then through an intramolecular aryl coupling, to form the 1,4,6,7,9,12-hexahydroperylene-3,10-quinone (HPQ) melanin (Fig. 3).[103] For many of these biaryl ring couplings, it is still unclear whether they utilize radical or cationic mechanisms and whether both substrates form radicals before coupling or one attacks the neutral distal substrate.

2.3.4 Other transformations. The versatility of P450s is on display in *Streptomyces* natural product biosynthetic pathways. PenM (CYP161C3) and PntM (CYP161C2) from *Streptomyces exfoliatus* and *arenae*, respectively, catalyze a rare oxidative rearrangement in pentalenolactone biosynthesis (Fig. 3).[113,114] After initial substrate radical formation, steric hindrance of the C-1 radical by substituents on the substrate itself severely suppresses oxygen rebound. This reduced rebound rate allows the oxidative formation of a neopentyl carbocation intermediate, which undergoes a 1,2-methyl shift followed by deprotonation to yield pentalenolactone.[114]

P450s can install oxygens on unactivated carbons and form ether linkages with pre-installed oxygens. Understandably, some P450s are able to do both. AurH, a P450 from *Streptomyces thioluteus*, catalyzes stereoselective tetrahydrofuran ring formation in aureothin biosynthesis (Fig. 3).[115–118] Two C–O bonds are sequentially formed: the first at the methylene C-7 to yield a 7*R*-hydroxy intermediate and the second between the newly installed hydroxyl and methyl C-9a.[116,117] It is unknown whether the heterocyclization steps utilize a transient dihydroxylated or radical intermediate. A similar mechanism may be used by AveE (CYP171A1) to form the tetrahydrofuran ring in avermectin.[119] PtmO5 also catalyzes tetrahydrofuran ring formation in platensimycin biosynthesis (Fig. 3).[120] PtmO5 likely stereoselectively hydroxylates the C-11 position (11*S*) on the *ent*-kauranol scaffold, then uses the pre-installed *R*-hydroxyl group at the C-16 position as a leaving group to form the 11*S*,16*S* ether ring.[120]

P450s are also able to form C–N bonds using at least two different strategies. Hemoproteins such as P450s can generate reactive nitrogen species, although reaction of molecular oxygen with heme-bound nitric oxide (NO) can cause irreversible enzyme inhibition.[121] TxtE, a P450 found in several plant-pathogenic *Streptomyces* spp. responsible for the production of thaxtomins, nitrates free L-Trp at the C-4 position (Fig. 3).[122–125] The proposed mechanism differs from typical Cpd I chemistry diverting after ferric superoxide (Fe^{III}–OO$^-$) is formed. Reaction of NO with ferric superoxide forms ferric peroxynitrite (Fe^{III}–OONO), which then undergoes heterolytic cleavage to yield NO_2 and Fe^{IV}=O followed by nitration and a Fe^{IV}=O-mediated hydrogen abstraction.[122] Nitration may also occur *via* electrophilic aromatic substitution if heterolytic cleavage is achieved through protonation of the ferric

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peroxynitrite complex.[122] The second strategy for C–N bond formation is the oxidative formation of an electrophilic functional group, which can subsequently be attacked by a nearby nitrogen atom. HmtT is responsible for hexahydropyrroloindole formation in himastatin going through a putative 2,3-epoxyindoline intermediate.[95,126] The nitrogen atom then attacks C-2 forming a C–N bond and leaving the hydroxyl group on C-3 (Fig. 3). Similarly, StaN (CYP244A1) may form the glycosidic bond in staurosporine *via* hydroxylation at C-5 of the sugar, followed by protonation and dehydration to yield the electrophilic oxonium cation (Fig. 3).[127,128]

In a unique C–S bond forming reaction, SgvP, from the griseoviridin biosynthetic pathway, forms a nine-membered thioene-containing lactone ring (Fig. 3).[129,130] Although only speculated at this time, the epoxidation of an α,β-unsaturated amide may lead to sulfur nucleophilic attack at the α carbon,[130] mirroring the proposals for C–N bond formation described above.

2.3.5 Xenobiotic transformations. Although this review mainly focuses on P450s in natural product biosynthesis, we would be remiss to not discuss the inherent role that P450s play in the oxidative transformations of xenobiotics. Given their associations with compound degradation and steroid biosynthesis, researchers have utilized P450s from *Streptomyces*, as well as other sources, as a tool to convert target compounds into compounds of interest. Notable examples include (i) a systematic screening of P450s for the regio- and stereoselective hydroxylation of testosterone,[131] (ii) the biosynthetic conversion of avermectin to 4′-oxo-avermectin for the agriculturally important insecticide emamectin,[44,132,133] and (iii) the multipurpose P450$_{SU-1}$ (CYP105A1), which, among other reactions, catalyzes the conversion of vitamin D_3 into its active form 1α,25-dihydroxyvitamin D_3,[134–136] O- and N-dealkylations of coumarin and sulfonylurea herbicides,[36,37,137,138] and the hydroxylation and epoxidation of diterpenoid-based resin acids.[139] In many cases, P450s with "xenobiotic" activities have unknown endogenous substrates and functions (*i.e.*, orphan P450s).

2.3.6 Non-P450 transformations. It is not unusual for proteins of similar sequence and/or function to perform vastly different types of chemistries with the triosephosphate isomerase (TIM) barrel fold being the classic example.[140] Proteins that are homologous to P450s in sequence and structure but catalyze unrelated reactions are beginning to be discovered. It has yet to be determined how prevalent these enzymes are in nature and in natural product biosynthesis.

In the biosynthesis of the methymycins and pikromycin in *Streptomyces venezuelae*, the glycosyltransferase (GTase) DesVII attaches TDP-desoamine to 10-deoxymethynolide or narbonolide.[141] However, DesVII requires a protein partner for proper activity. DesVIII, a protein with a P450-like sequence minus the conserved Cys, was shown to have protein–protein interactions with DesVII and improve DesVIII activity at least 1000-fold.[141–143] EryCII, a homologue of DesVII from the erythromycin biosynthetic pathway in *Saccharopolyspora erythraea*, was recently confirmed to have the P450-like fold and allosterically activates the GTase EryCIII by forming a homotetramer.[144] By analogy, DesVIII and its homologous non-heme containing P450-like proteins are allosteric activators of macrolide GTases.

CYP170A1 catalyzes two sequential and nonstereoselective allylic oxidations in the conversion of the sesquiterpene epi-isozizaene into the antibiotic albaflavenone in *S. coelicolor* (Fig. 3).[145] Interestingly, CYP170A1 generated isomers of farnesene, farnesol, and nerolidol in a redox-independent manner when incubated with farnesyl diphosphate (FPP).[146] A second active site on the P450 scaffold was found, mimicking a terpene synthase in both sequence and structure.[146] Homologues of CYP170A1, such as CYP170B1 from *Streptomyces albus*, do not have the terpene synthase functionality, likely due to the inability to bind Mg^{2+} given its DGXXR motif, instead of the canonical DDXXD terpene synthase motif.[147] It is unclear if the moonlighting terpene synthase function of CYP170A1 is biologically relevant.

3 Sequence

It is well known that the P450 superfamily exhibits significant sequence heterogeneity and lacks conserved sequence motifs. Until 2006, there were only three residues that were thought to be strictly conserved among all of the P450 sequences in the public databases (Fig. 4). The first invariant residue, and catalytically most important, was a Cys, the fifth axial ligand of the heme iron.[148] The other two invariant residues were the Glu and Arg that composed the EXXR motif found in the K-helix.[149] These two charged residues form ionic interactions with the region prior to the Cys-ligand loop (meander region) to assure correct tertiary structure and stabilization of the heme within the protein.[150] Once the genomes of *S. avermitilis* and *S. coelicolor* were available, it was clear that there were P450s in nature that did not possess the EXXR motif.[31,53] CYP157C1 from *S. coelicolor* was later confirmed to be a legitimate heme-containing P450, indicating that only the iron-binding cysteine is strictly conserved.[151] There are two other highly conserved, but nonessential, residues found in P450s: a Thr in the I-helix, which has been proposed to be involved in oxygen activation[152] and the prevention of auto-inactivation,[153] and a Gly four residues upstream of the conserved Cys, which allows the formation of the β-hairpin turn.[150] Sequence alignment of the 184 (minus CYP102D1 due to its significantly longer sequence) functionally characterized P450s from *Streptomyces* reveals the highly conserved nature of the EXXR motif and the heme-binding Cys, Thr, and Gly residues (Fig. 4). Although no residues are completely conserved within these P450s, there are indeed several highly conserved motifs. HXXXR in the C-helix, Arg in β5, and Asp, Arg, and Phe in the meander region. Many of these residues comprise the heme-binding pocket; however, the roles of other conserved motifs outside of the active site is unknown. These residues may contribute to the structural integrity of the P450 fold or protein–protein interactions with redox partners.

Interestingly, as more P450 sequences become available, even the heme-binding Cys paradigm is being challenged. CYP107AJ1 from *Streptomyces peucetius* does not contain the expected Cys, yet was shown to be a legitimate P450 that retains peroxygenase activity.[154] Other natural variants of P450s without

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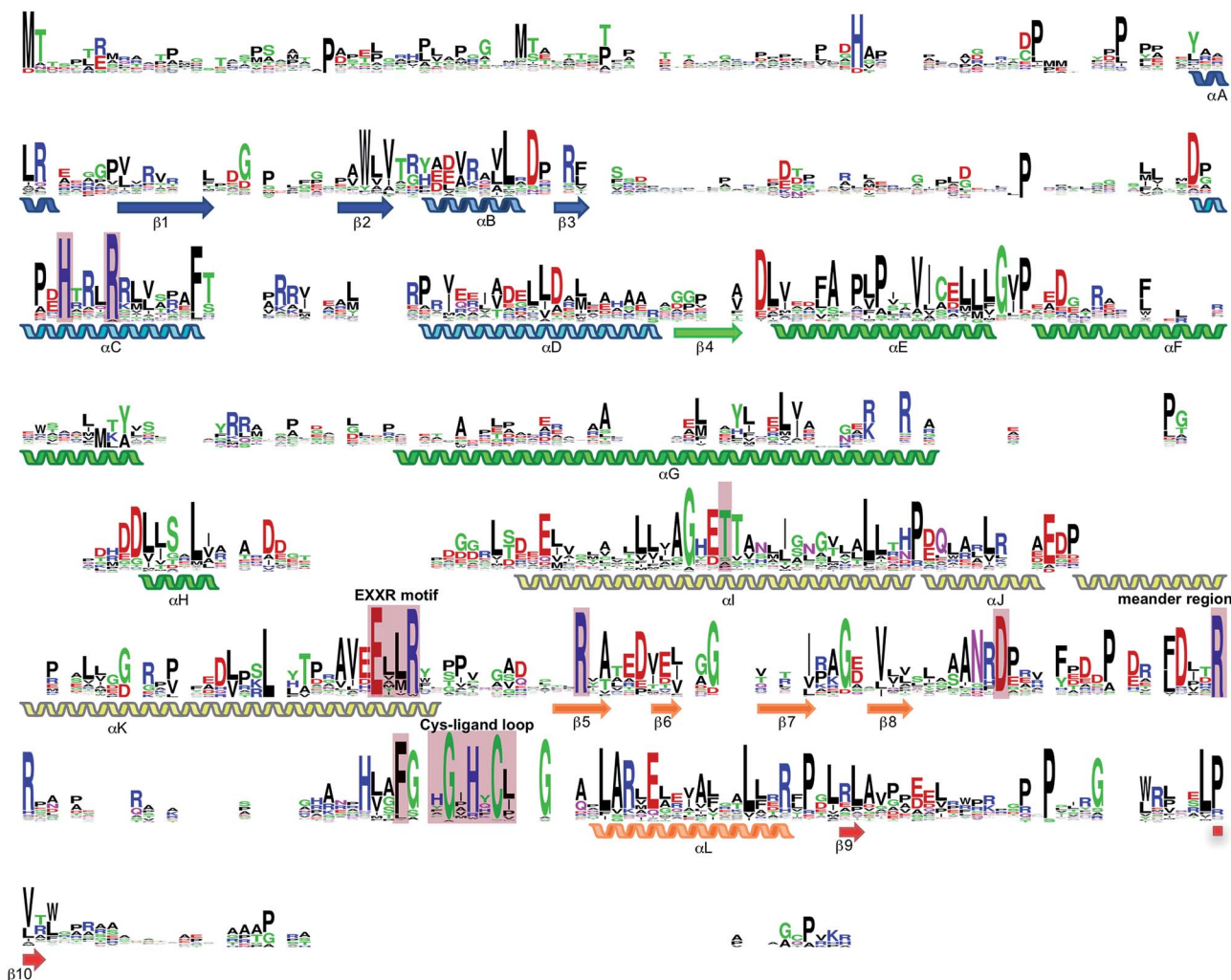This journal is © The Royal Society of Chemistry 2017

Fig. 4 Sequence alignment of the 184 functionally characterized P450s from *Streptomyces* (minus CYP102D1, for simplicity, given its extended length). The heme-binding Cys, EXXR motif in the K-helix, and Thr in the I-helix are highly conserved. In addition, there are other highly conserved motifs and residues, both within and outside of the active site, in *Streptomyces* P450s. Residues discussed in the text are highlighted by pink boxes. Positions with no residues represent gaps in the alignment due to sequence length differences.

the axial Cys have also been identified in other organisms, although their functions are still unknown.[155] Engineered P450s with Cys-to-Ser or Cys-to-His mutations have also been shown to promote unnatural carbene- and nitrene-transfers, as well as peroxidase activity,[156–159] suggesting that natural variants without the conserved Cys may still in fact be functional P450s. Finally, as described in Section 2.3.6, there are P450-like proteins, *e.g.*, GTase activator DesVIII and its homologues, that lack both the axial Cys and heme,[143,144] indicating that P450s are evolving to utilize the P450 scaffolding for alternate functions.

Given the amino acid sequence disparity among P450s, as well as our limited understanding of P450 sequence–structure–function relationships, it is currently impossible to predict P450 function or substrate preference based on sequence alone. However, with the enormous influx of bacterial genomes, we set out to map the current catalytic landscape of P450 enzymes of streptomycete origin.

3.1 Network generation

P450 protein sequences, limited to the taxonomic order of Streptomycetales, were obtained from the InterPro database.[160] As of January 3, 2017, there were 8578 P450 sequences; of these, 7818 were unique (*i.e.*, <100% identity). To emphasize the rapid rate at which the number of P450s in the database are increasing, there were 6722 P450 sequences of streptomycete origin on September 15, 2016, corresponding to an increase of >18 new P450s per day! Given this extraordinary rate of growth, a systematic approach to prioritizing P450s is necessary, along with innovative high-throughput methods, for focused and informative future studies.

We used 8579 P450 sequences (the 8578 sequences from InterPro plus SoCYP158A2, as it was not found in the InterPro database) and generated a P450 sequence similarity network (SSN)[161] using the Enzyme Function Initiative-Enzyme Similarity Tool (EFI-EST).[162] After using a minimum length cutoff of 200 amino acids and a representative node network of 100%

identity, there were 7579 unique P450 sequences. Of these, 184 (~2.4%) have been functionally characterized (Table 1). To select an E value threshold for the network, we attempted to parallel the systematic CYP nomenclature (*i.e.*, family and subfamily >45% and >55% identity, respectively)[163] to give the community a valid point of reference. As the networks are based on E values, which take sequence length into consideration, but the CYP nomenclature are based on sequence length-independent percent identities, we used the functionally characterized P450s that have corresponding CYP names as a guide for threshold selection. For the CYP family level, we selected a threshold of 10^{-85} to prevent HlsI[164] from separating from CYP107 family. For the CYP subfamily level, we selected a threshold of 10^{-124} to keep ComJ[165] within the CYP165B subfamily. The E values of 10^{-85} and 10^{-124} are approximately equivalent to median values of 45% and 58%, respectively, for P450s with 400 residues. Access to the P450 SSN is available at http://www.scripps.edu/shen/NPLI/database.html.

3.2 Pre-CYP family network

Before separating the P450s into their approximate CYP family and subfamily networks, we evaluated the global organization of streptomycete P450s. At an E value of 10^{-35} (low stringency threshold), the two main classes of P450s can already be distinguished. These two major clusters of proteins represent the bacterial and mitochondrial soluble class I and the eukaryotic microsomal membrane-bound class II (described in Section 2). The larger class I subtype contains >2300 unique P450 sequences and includes the vast majority of the functionally characterized enzymes. The smaller class II subtype, which contains the self-sufficient CYP102D1 and the CYP170 family, is comprised of <400 unique P450s. In addition to the two major clusters, there are a handful of smaller clusters revealing P450 or P450-like proteins that are significantly different in sequence from the two main classes. All but one of these small clusters only contain uncharacterized members. The only known P450 included in this group is CYP155A1, a legitimate P450 with an unknown endogenous function.[31] Conspicuously, the P450-like GTase activators already show signs of separation from the class I cluster at this threshold.

As in all SSNs, as the similarity threshold is increased, differences in P450 sequences become more apparent. In general, before the CYP family cutoff of 10^{-85} is reached, the large clusters begin to separate into what will become the CYP family clusters. One of the most diverse P450s that resolves into an independent cluster early is the C–N bond forming StaN,[100,127,128] perhaps indicative of its CYP244 designation. Interestingly, a cluster of sequences that starts to splinter off the class I subtype at 10^{-45} and has clear separation at 10^{-55} contains only P450s that work on ACP- or PCP-tethered substrates including MonD,[79] NovI,[166] NikQ,[167,168] SanQ,[169] Qui15,[170] and P450$_{sky}$.[76–78] This finding suggests that this cluster of P450s (>180 members) may all work on ACP- or PCP-tethered substrates and gives insight into the putative endogenous function of CYP125A2, a P450 shown to hydroxylate a xenobiotic flavonoid.[171] Not all characterized P450s that act on ACP- or

PCP-tethered substrates are found within this cluster, with AcmG8,[172] StaF, and StaH being notable exceptions,[99,106] suggesting there is more than one type of sequence that can utilize protein–protein interactions for substrate selectivity.

3.3 CYP family and subfamily networks

While we used the systematically named and functionally characterized P450s from *Streptomyces* as a guide for determining the CYP family network found in this review, it is clear that the determined E value of 10^{-85} is not perfect in separating members of different CYP families from each other. There are >100 clusters with at least five unique P450 sequences and >300 smaller clusters or singletons (Fig. 5). Parenthetically, bacterial P450s encompass CYP100–CYP2999;[57] although it is not clear how many are found within *Streptomyces*. While many of these clusters are separated into different CYP families, there are exceptions. In addition, the diversity and growing number of new P450 sequences results in common neighbors of CYP families, linking these clusters together. As with the CYP nomenclature, one SSN threshold will never be perfect; however, utilizing a range of thresholds likely gives the best opportunity to systematically categorize such a large number of protein sequences.

After the CYP family network was annotated, depicting P450 function, substrate (type of natural product scaffold), and functional characterization by color, shape, and label of each node, respectively, several conclusions are immediately apparent. (i) Streptomycetes possess a great diversity of P450s, most of which have not been studied. More than 75 clusters with at least five unique members do not have a functionally characterized P450, and that does not include the >50 P450s with unknown endogenous functions. (ii) Large CYP family clusters give an impression of conservativity across different *Streptomyces* species, suggesting these may be involved in much less specialized roles, *e.g.*, detoxification. The largest supercluster is composed of both the CYP105 and CYP107 families, the two largest CYP families in bacteria.[4] Smaller clusters and singletons, P450s that are not present in many different species, likely have evolved specific functions for less common biosynthetic pathways or substrates. (iii) At this similarity level, there are no overall tendencies of P450s of similar function or preferred substrate to cluster together. For example, not all ether-forming P450s cluster together, with AurH,[115–118] AveE/MeiE,[119,173,174] and PtmO5 (ref. 120) existing in three different clusters. This corresponds to the difficulty in predicting P450 function based on overall P450 sequence.

(iv) From a more local perspective, there are some clusters that show similarities in function and/or substrate preference. As described above, a group of P450s that act on ACP- and PCP-tethered substrates showed early separation from the larger class I cluster. At an E value of 10^{-85}, the ACP-dependent MonD[79] separated from a group of 145 P450s containing only known enzymes that hydroxylate PCP-tethered amino acids at the β position, *i.e.*, NovI,[166] NikQ,[167,168] SanQ,[169] Qui15,[170] and P450$_{sky}$.[76–78] Their separation from other P450s, combined with their sequence similarities suggest that they possess sequence–
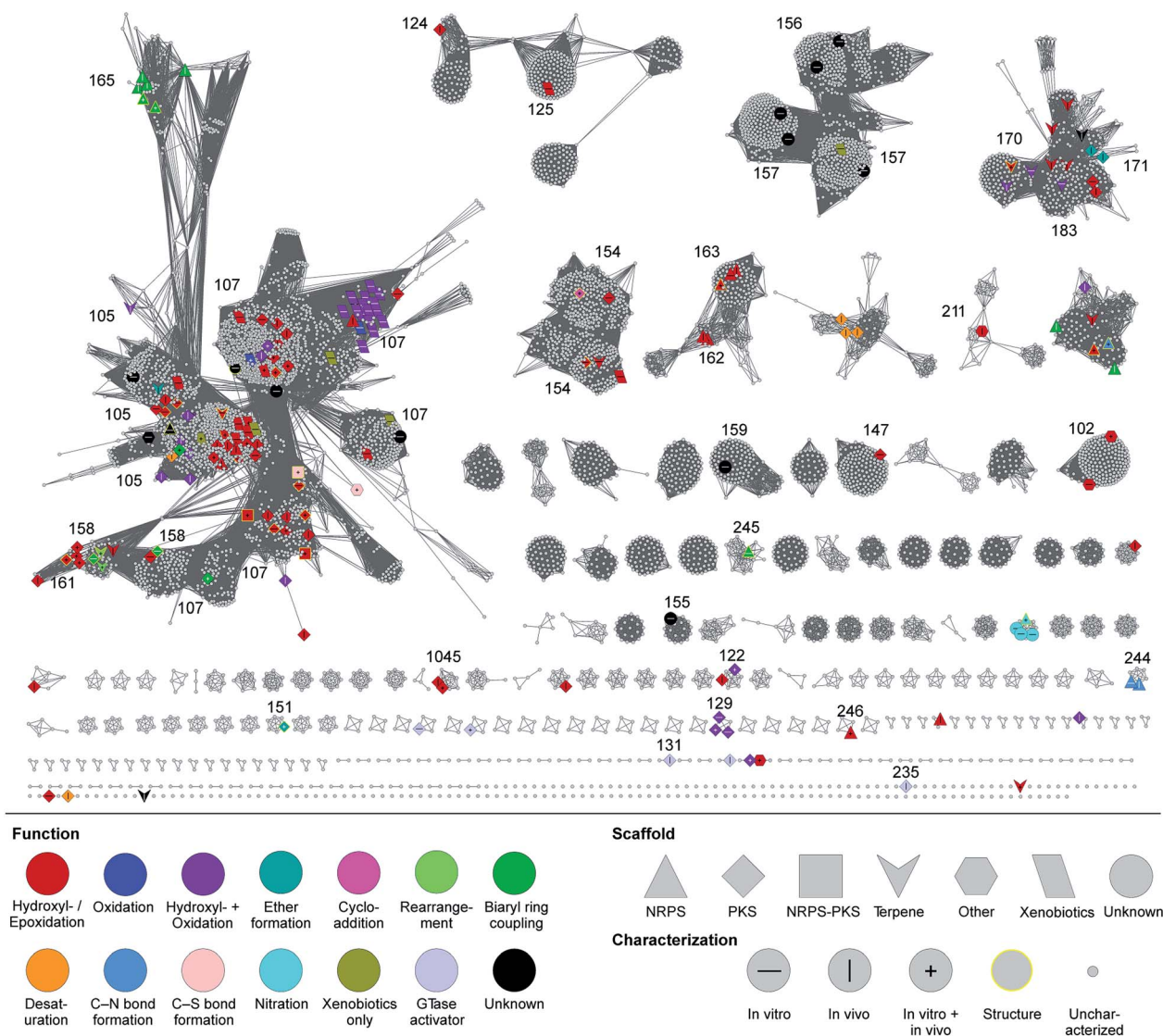
Fig. 5 CYP family SSN of *Streptomyces* P450s. The SSN is shown at a BLAST *E* value cutoff $= 10^{-85}$ (median 45% identity over 400 residues). Larger nodes are functionally characterized P450s with node labels describing how it was characterized. Colors and shapes of nodes represent P450 function and substrate type (type of natural product scaffold). See inset legend for details. CYP families of functionally characterized P450s are labeled.

function or sequence–substrate relationships. Although the amino acid specificities differ amongst these P450s, the P450–PCP (or P450–phosphopantetheine) interactions are likely responsible for substrate recognition. Several predicted, but uncharacterized, P450s involved in the biosyntheses of β-amino acid containing natural products such as simocyclinone,[175] clorobiocin,[176] and courmermycin,[177] also cluster in this group. Finally, (v) the biaryl ring coupling-catalyzing P450s involved in glycopeptide biosynthesis, are splintering off of the CYP105 family. These include the aforementioned StaH, StaG, StaF, and StaJ[99,106] and ComI and ComJ from complestatin biosynthesis.[165] Although only StaH and StaF were characterized *in vitro* showing a requirement for the PCP-tethered substrate and the X-domain,[107] it is plausible that the other four enzymes, along with the other unidentified members in this splintered group follow the same reactivity requirements.

The CYP subfamily network, with an *E* value of 10^{-124}, significantly increases the level of complexity within the P450 network (Fig. 6). The number of clusters with at least five unique P450 sequences, clusters with less than five, and singletons inflated to >200, >300, and >500 respectively. As with the lower threshold network, there are a few exceptions to our attempt to limit one subfamily per cluster. Importantly, and in opposition to the CYP family network, nodes of similar colors and/or shapes, *i.e.*, functionally characterized P450s with similar functions and/or substrates, cluster together more frequently. For example, AmphN,[178,179] NysN,[180,181] and ScnG,[182,183] P450s that all convert a methyl group into a carboxylic acid in the biosynthesis of polyenes amphotericin B, nystatin, and pimaricin, respectively, cluster together into one subfamily (CYP105H). TxtE, the tryptophan-nitrating enzyme[122–124] clusters together with seven other P450s, including the three P450s that
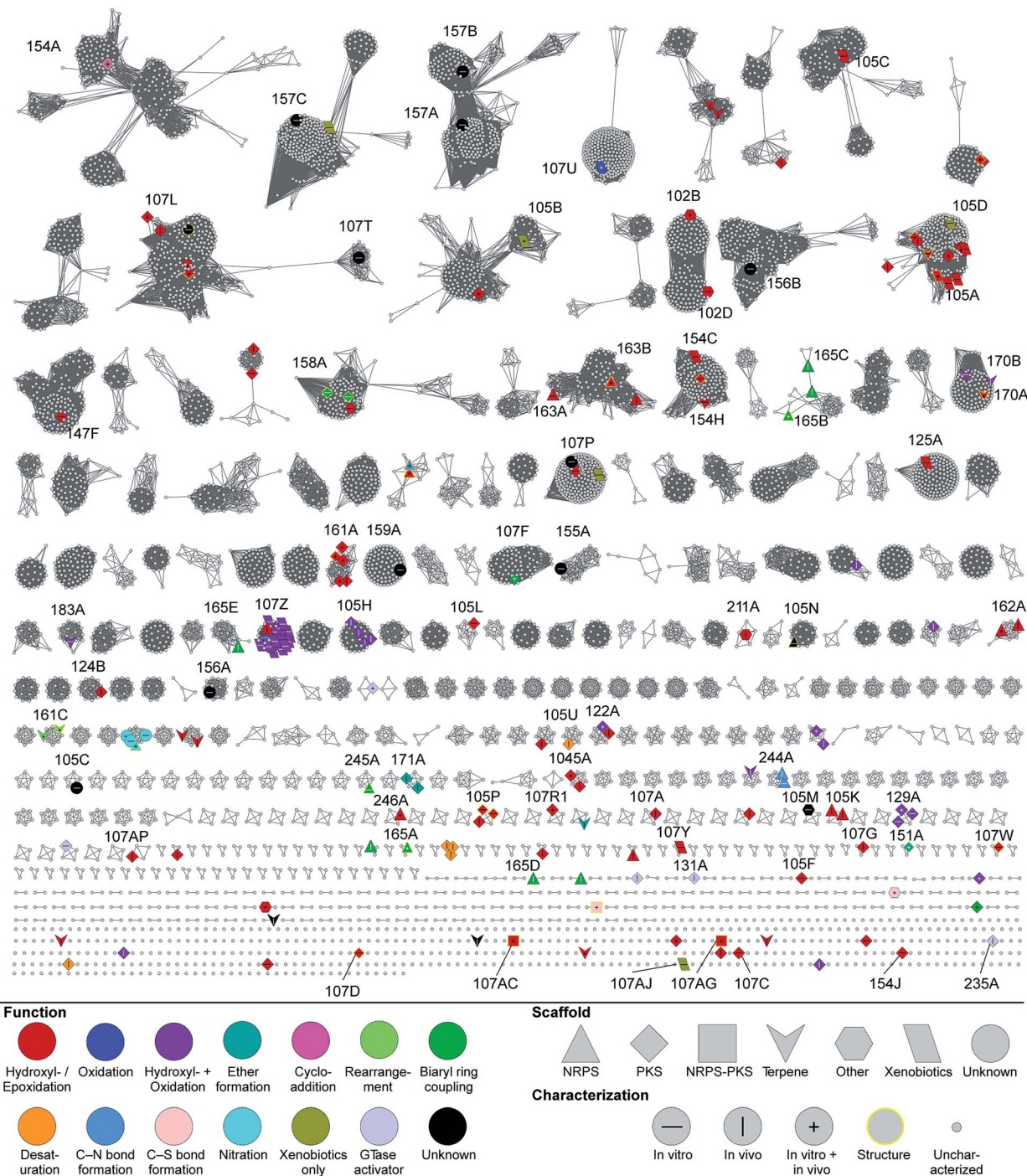
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Fig. 6 CYP subfamily SSN of *Streptomyces* P450s. The SSN is shown at a BLAST *E* value cutoff $= 10^{-124}$ (median 58% identity over 400 residues). Larger nodes are functionally characterized P450s with node labels describing how it was characterized. Colors and shapes of nodes represent P450 function and substrate type (type of natural product scaffold). See inset legend for details. CYP subfamilies of functionally characterized P450s are labeled.

were identified to nitrate the C-5 position on the indole ring of tryptophan.[125] Even NikQ[167,168] and SanQ,[169] the PCP-dependent β-hydroxylating P450s separate from the other group of PCP-dependent β-hydroxylating enzymes. This may indicate sequence differences that may account for PCP specificity, or alternatively, a difference in amino acid preference. The most striking revelation in this CYP subfamily network is the

immensity of unknown and uncharacterized P450s found within both large and small clusters.

Overall, the P450 SSN clearly emphasizes (i) the sequence diversity of P450s found within *Streptomyces*, (ii) that higher levels of sequence similarity, in general, can be used to cluster P450s of similar tendencies, and thus identify clusters of novel, uncharacterized P450s, and (iii) the majority of these clusters

contain no functionally characterized members. The identification of the most promising P450s and their functional and structural characterizations will surely expand the chemical reaction space of P450s found in the biosynthesis of natural products.

4   Structure

Since the first crystal structure of a P450, P450$_{cam}$ (CYP101A1) from *P. putida*, was determined in 1985,[184] the rate of P450 structures has dramatically increased every year. At the time of writing this review, there are around 750 P450 structures deposited within the Protein Data Bank (PDB). Twenty-nine of these are from *Streptomyces*, with 13, eight, three, and four coming from polyketide, nonribosomal peptide, hybrid polyketide–nonribosomal peptide, and terpene biosynthesis, respectively; one is still functionally unknown. In the CYP family SSN, less than 15 clusters with at least five unique members have at least one PDB entry, revealing a significant lack of CYP families and subfamilies that have been structurally characterized. In this section, we describe the general structure of P450s, structures from *Streptomyces* that catalyze interesting chemistries or give mechanistic insight into this family of enzymes, and examples showing the potential of structure-based engineering of P450s. While other reviews have covered the topic of P450 structure,[3,16,185] we aim here to highlight those from *Streptomyces*.

4.1   Common structural aspects

There are now a sufficient number of P450 structures from eukaryotes, bacteria, and archaea sources to verify that all P450s share similar two- and three-dimensional structures, even in spite of low sequence identities between some members.[185] The fold and overall structures of the 29 P450s from *Streptomyces* are nearly identical, particularly the protein architecture surrounding the heme binding site. The root-mean-square deviations (RMSD) of the 29 structurally aligned P450s ranged from 1.3 Å to 2.6 Å, with an average value of 1.8 Å. The P450s from *Streptomyces* mainly consist of 12 α-helices (αA–αL) and 10 β-strands (β1–β10) and fold into prism-like structures (Fig. 4 and 7A). As in all P450s, the P450s from *Streptomyces* feature an extended I-helix passing through the length of the protein. The binding environment of the heme prosthetic group is quite conserved, being sandwiched between the I-helix and Cys-ligand loop. The Cys-ligand loop forms a rigid architecture *via* two main chain H-bonds, which positions the conserved Cys at an ideal place for coordination with the heme iron. A highly conserved Phe (Fig. 4), seven amino acids prior to the conserved Cys, interacts with the heme *via* T-shaped π–π stacking. Two His (C-helix and Cys-ligand loop) and two Arg (C-helix and β5 helix), both highly conserved residues, (Fig. 4) bind to the two propionate groups of heme *via* electrostatics interactions. Superposition of the 29 P450 structures revealed both regions of strict alignment and variability (Fig. 7C). Regions that were most structurally conserved were the I- and L-helices and the Cys-ligand loop; regions showing subtle differences between

structures were mainly in the N-terminal loop, A–B and B–C loops, and F–G region (including the F–G loop and the F- and G-helices). In general, the substrate binding site of P450s is located near the B–C and F–G loops, helices F, G, and I, and the heme. The B–C loop (in some P450s, contains 1–3 small helices) and F–G region form the active site entrance and are responsible for substrate recognition. The B–C loop and F–G region undergo a conformational change, from an open to closed state, upon substrate binding (Fig. 7B).[185] However, the conformations of some P450s are not controlled by substrate binding. For example, the crystal structure of substrate-free PikC (PDB ID: 2BVJ) was shown to exist in both open and closed conformations within the two polypeptide chains.[186]

4.2   Variations in structure

As described above, the 29 P450 structures from *Streptomyces* are highly conserved. Given the inherent ability of P450s to perform diverse chemistries on diverse substrates, there must be structural variations between P450s that account for these differences. The same concept is found in the SSN, where there is not a universal pattern for sequence–function relationships, but local trends do exist. Since structure is typically determined based on sequence (with the exception of convergent evolution of protein folds), the P450 SSN gives insight into finding P450s with unique structural properties. For example, the PCP-dependent P450s, which show sequence similarity, likely have sequence-associated structural similarities due to their requirement for PCP-P450 protein–protein interactions. Another interesting observation is that none of the P450s found within the cluster containing the EXXR motif-less CYP157C1 (264 members in total) possess the EXXR motif, supporting that P450s with similar sequences have similar structural motifs. No members within this cluster have been structurally characterized yet. The structures of selected P450s from *Streptomyces* with unusual biochemical properties are presented in this section.

4.2.1   Heme group inversions. Almost all P450s incorporate the prosthetic heme group in the same orientation, based on the positions of the two protoheme vinyl groups. A crystal structure of CYP121A1 (PDB ID: 1N40) from *Mycobacterium tuberculosis* revealed that the heme group can be in two distinct orientations, the normal orientation and one in which the heme is flipped 180°.[187] The crystal structure of CYP154A1 (PDB ID: 1ODO) from *S. coelicolor* revealed that its heme group only resides in the 180°-flipped orientation (Fig. 8A).[188] CYP154A1 catalyzes an unusual redox-independent Paternò–Büchi-like [2 + 2] cycloaddition of a dipentaenone into a oxetane-containing product.[30] The role of this natural product or intermediate is not clear, but deletion of the CYP154A1-encoding gene compromised the stability of the bacterial spores of the producing strain.[30] Three additional structures of P450s from *Streptomyces*, StaF (PDB ID: 5EX8),[106] SgvP (4MM0),[189] and CYP105P2 (5IT1),[190] revealed that their heme groups also adopt this unusual flipped orientation (Fig. 8A). It remains unclear whether heme group orientation is the consequence of protein sequence or a distinct incorporation process during P450 folding, and how, if at all, it affects the structure or catalytic
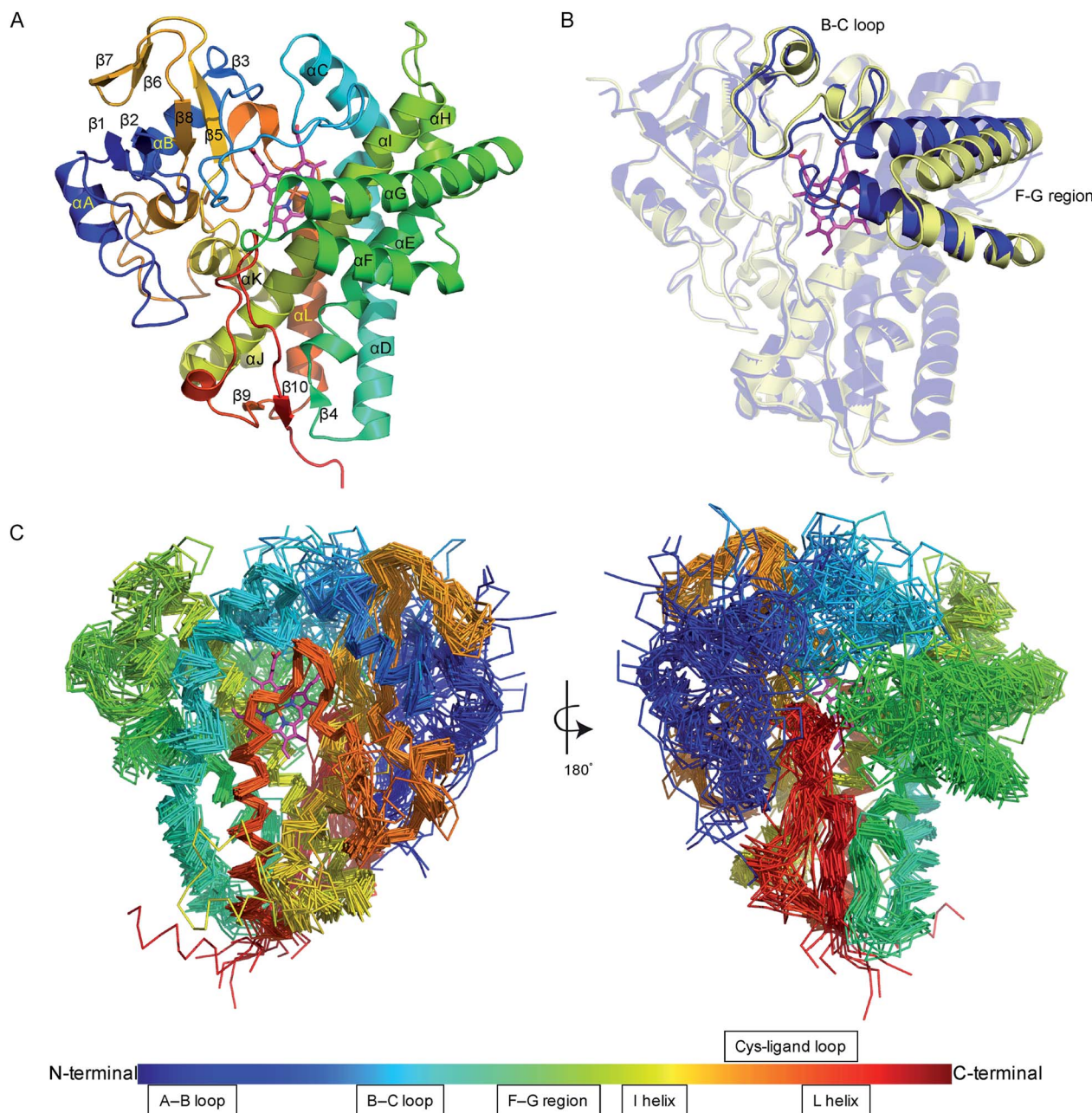
Fig. 7 Common structural aspects of P450s from *Streptomyces*. (A) The overall structure of P450s (exemplified by PDB ID: 3ABA). (B) The open and closed states of the P450 active site are facilitated by conformational changes in the B–C loop and the F–G region [PDB IDs: 1SE6 (open) and 2D09 (closed)]. (C) Structural superposition of the 29 structurally characterized P450s from *Streptomyces* highlighting the regions of high, moderate, and low structural conservativity.

mechanism of the P450. The four P450s containing this unusual flipped heme group did not cluster together in the SSN at either the CYP subfamily or family level.

4.2.2 Carrier protein recognition. In some instances, P450s recognize substrates that are tethered to carrier proteins found in natural product biosynthetic assembly lines. P450$_{sky}$ catalyzes β-hydroxylation of three PCP-tethered amino acids in the biosynthesis of skyllamycin.[76–78] The crystal structure of P450$_{sky}$ revealed a typical P450 fold (PDB ID: 4L0E, 4L0F, 4PWV, 4PXH).[77,78] The complex structure of P450$_{sky}$ and an inhibitor-

tethered PCP domain from module 7 revealed that the interfaces of P450$_{sky}$ and PCP form several hydrophobic interactions and a few electrostatics interactions (Fig. 8B).[78] In contrast to other typical P450s, the structure of P450$_{sky}$ in the P450–PCP complex only shows differences to that of unbound P450$_{sky}$ in the interface of the F- and G-helices; the B–C loop of P450$_{sky}$ does not rearrange upon binding to the PCP. Furthermore, the structure of P450$_{sky}$ exhibits an unusual additional C-terminal M-helix (Fig. 8B), which may serve to mediate interactions with either the redox partner proteins of P450$_{sky}$ or other NRPS
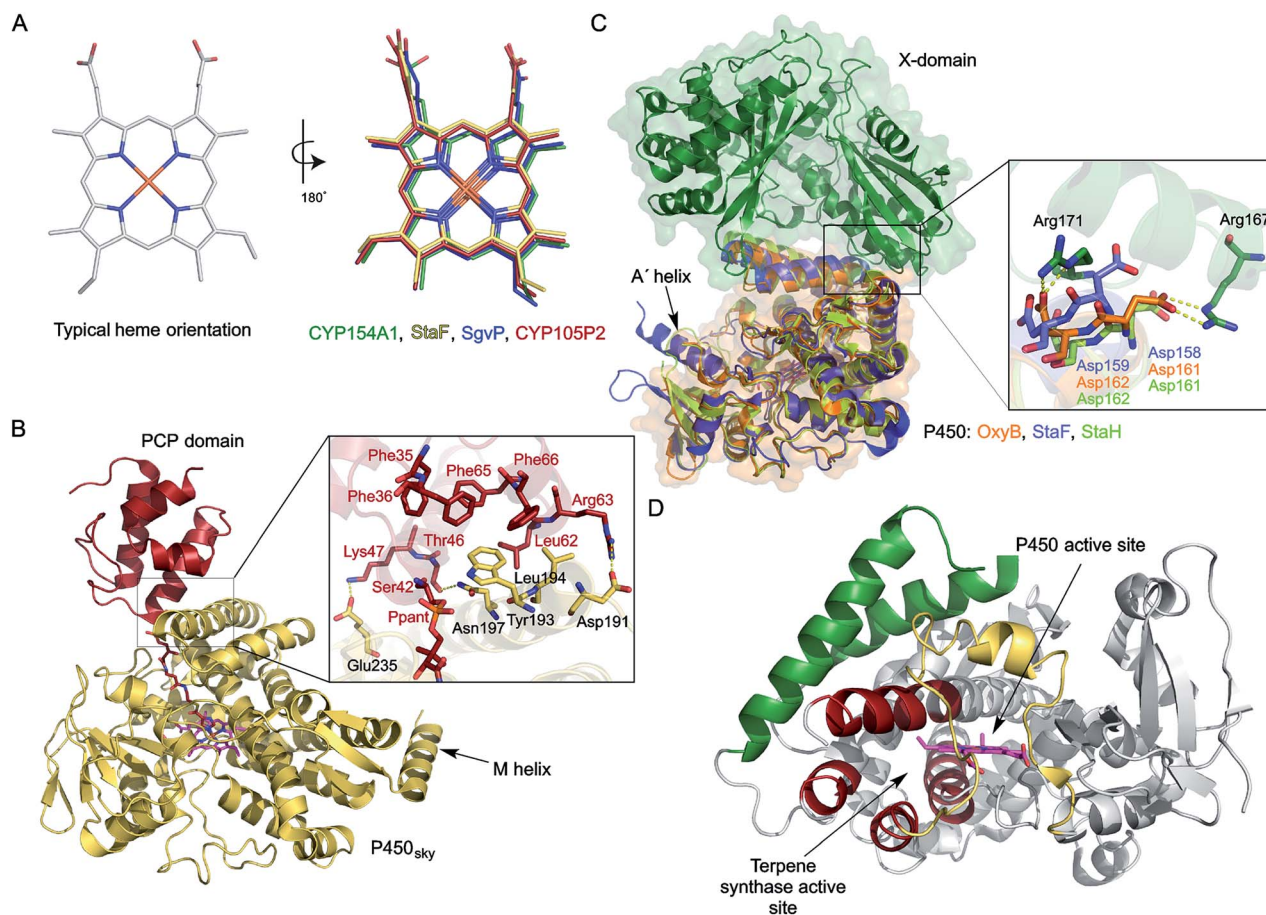
Fig. 8 Selected variations in the structures of P450s from *Streptomyces*. (A) Typical heme orientations and inversed orientations in four different P450s (PDB IDs: 1ODO, 5EX8, 4MM0, 5IT1). (B) The crystal structure of P450$_{sky}$ in complex with an inhibitor-tethered PCP domain (PDB ID: 4PXH). The inset shows a zoomed-in look at the interface of P450$_{sky}$ and the PCP domain highlighting the hydrophobic and electrostatic interactions. The unusual C-terminal M-helix is labeled. (C) Structural superposition of StaF (PDB ID: 5EX8) and StaH (PDB ID: 5EX6) with OxyB in complex with the X-domain (PDB ID: 4TX3). The inset shows the two Asp residues from the conserved PRDD motif, which are proposed to be involved in recruitment by the X-domain *via* interaction with two Arg residues from the X-domain. (D) The crystal structure of CYP170A1 (PDB ID: 3EL3). The B−C loop and F−G region, colored in yellow and green, respectively, form the P450 active site. The four helices, colored in red, form the terpene synthase active site.

domains in close spatial proximity to the PCP domain (Fig. 8B).[77] P450$_{sky}$, which hydroxylates PCP-tethered amino acids in modules 5, 7, and 11 of the skyllamycin NRPS, did not show any interaction with either the PCP domain from module 10 of the skyllamycin NRPS or a heterologous ACP from *E. coli*,[78] indicating that P450$_{sky}$ recognizes specific carrier proteins.

4.2.3 P450 recruitment by an NRPS X-domain. The teicoplanin-like GPA A47934 involves four P450s that not only accept PCP-tethered substrates, but also interact with the X-domain for P450 recruitment and cyclization efficiency.[97,98,107] The biosynthetic gene cluster of A47934 possesses four genes encoding P450s, StaH, StaG, StaF and StaJ, which were demonstrated to participate in C−O−D, F−O−G, D−O−E, and A−B ring formations, respectively.[99,106] The X-domain was demonstrated to recruit StaF and StaH to catalyze moderate levels of PCP-tethered peptide cyclization.[106,107] The interaction between the P450 and X-domain was first identified based on the crystal structure of OxyB in complex with the X-domain in the teicoplanin system (PDB ID: 4TX3) (Fig. 8C).[191] OxyB binds to the

X-domain through interaction with the D-, E-, F- and G-helices. Two Asp residues in a PRDD motif, which is conserved in the F-helix within all GPA crosslinking P450s, contact two conserved Arg residues from the X-domain. The crystal structures of StaF (PDB ID: 5EX8) and StaH (PDB ID: 5EX6) were also recently solved, revealing typical cytochrome P450 structures and high structural homology with OxyB (RMSD of 0.7 Å and 1.2 Å, respectively).[106,107] As in OxyB, the F helix PRDD motifs of StaF and StaH are proposed to be involved in recruitment by the X-domain (Fig. 8C). Furthermore, StaF, like its orthologue OxyA in teicoplanin biosynthesis, features a long A′ helix at the N-terminus, which is unique for D−O−E ring-catalyzing P450s in GPA biosynthesis (Fig. 8C).[106,192] However, the function of this extra helix remains unclear.

4.2.4 Moonlighting active site. CYP170A1 is an extremely unique P450. Not only is it a multifunctional monooxygenase that catalyzes two sequential allylic oxidations in the biosynthesis of albaflavenone,[145] it also has terpene synthase activity that converts FPP into a mixture of farnesene isomers. Within

its normal prism-like P450 structure, CYP170A1 possesses a novel terpene synthase active site (PDB ID: 3EL3, 3DBG).[146] This active site contains the Mg^{2+} binding motifs DDXXD and DTE that are typical of canonical terpene synthases. The terpene synthase active site is formed by a four helix α-helical barrel (helices C, H, I, and L), which is unusual compared to the six α-helices found in all other terpene synthases (Fig. 8D).[146] This is the first functional P450 that possesses another non-P450 active site that is moonlighting on the basic P450 structure. It is unknown if the other members of the CYP170 family, those without the DDXXD and DTE motifs and thus the terpene synthase activity, have the same four α-helical barrel. CYP170A1 appears to be an example of evolution caught-in-action, supporting that structural elements, beyond the heme binding site, in the P450-fold are conducive for evolving into other functions.

4.3 Structure-based mechanistic studies

The functional versatility of P450s is clearly evident. However, except for the commonly accepted mechanism for hydroxylation, the catalytic mechanisms of many of the unusual functions of P450s remain unclear. Crystal structures from natural product P450 enzymes, particularly those in complex with their endogenous substrates, help to investigate and understand substrate binding modes, protein conformational changes, and enzyme catalytic mechanisms. Here we present some examples of P450 crystal structures from natural product biosynthetic pathways in *Streptomyces* that shed light on some of the uncommon P450 reactions.

4.3.1 Epoxidation *via* the hydroperoxyferric intermediate.
PimD catalyzes epoxidation of the C-4–C-5 double bond in the polyene macrolide pimaricin.[50,70,71] The crystal structure of PimD in complex with its substrate, 4,5-desepoxypimaricin (PDB ID: 2XBK), revealed that the π-orbitals of the C-4–C-5 double bond of 4,5-desepoxypimaricin point away from the iron at an angle of ∼125° and are too distant for synchronous oxygen insertion by Cpd I (Fig. 9A).[50] In fact, the bond angle and distance (3.7 Å) appear to be favorable for a typical abstraction and rebound hydroxylation mechanism, but the high dissociation energy of abstracting a hydrogen from a vinyl carbon likely precludes it (Fig. 9A).[50] Alternatively, epoxidation by PimD may occur *via* a concerted substrate-assisted mechanism in which the hydroperoxoferric intermediate, Cpd 0, acts as the oxidant. The highly nucleophilic peroxoferric intermediate first abstracts a hydrogen from the C-7 hydroxyl of the substrate, then a concerted and cyclic six-electron rearrangement of Cpd 0 with the C-4–C-5 double bond inserts the distal oxygen atom yielding the epoxy product (Fig. 9A).[50] Evidence for Cpd 0 oxidation for the PimD reaction mechanism was further confirmed as the formation of pimaricin was detected when incubated with hydrogen peroxide, but not with organic peroxides that do not form Cpd 0.[50] If steric and/or electronic factors prevent interaction of Cpd I with the substrate, as in the case of PimD, the reactivity of other intermediates in the P450 catalytic cycle can be invoked to catalyze the desired reaction.

4.3.2 Substrate-assisted biaryl ring coupling.
CYP158A2 catalyzes C–C bond formation to polymerize flaviolins.[42,102,111,112]

CYP158A2 is a substrate-assisted enzyme, requiring its substrate to contain a proton donor or acceptor to stabilize water molecules as it does not contain the highly conserved Thr in the I-helix. The crystal structure of CYP158A2 contains two molecules of flaviolin in the active site cavity (PDB ID: 1T93, 2D09) (Fig. 9B).[102] The C-5 and C-7 hydroxyl groups of one molecule of flaviolin stabilize catalytically important water molecules that are involved in dioxygen activation (Fig. 9B).[111] Both diradical and cationic mechanisms have been proposed for this reaction. In the diradical mechanism, initial hydrogen abstraction is postulated to occur from the C-5 hydroxyl group (Fig. 9B). The radicals localize at C-3 or C-8 and subsequently undergo C–C coupling reaction. In the cationic mechanism, an initial *ipso* attack at C-5 yields a flaviolin–iron covalent intermediate and generates a positive charge at C-3. The carbon cation is then nucleophilically attacked by C-3 of the adjacent flaviolin molecule to from the C–C bond (Fig. 9B).[102,111]

CYP158A1 shares high sequence identity and structure similarity to CYP158A2. CYP158A1 also catalyzes the biaryl ring coupling reaction between flaviolin molecules, albeit with a different preferred regioselectivity compared to that of CYP158A2.[42] The major biflaviolin products of CYP158A1 and CYP158A2 are the C-3–C-3′ and C-3–C-8′ coupled products, respectively.[42] As in the CYP158A2 structure, two flaviolin molecules were found in the crystal structure of CYP158A1 (PDB ID: 2NZ5).[42] One flaviolin molecule is positioned over the heme group, similar to that found in CYP158A2; the second flaviolin molecule is bound at the entrance to the substrate access channel, which is too far from the first flaviolin for dimerization (Fig. 9B). Conformational changes were proposed to facilitate movement of the second flaviolin molecule towards the heme-adjacent flaviolin molecule.[42] Although it is still unclear whether CYP158A1 and CYP158A2 utilize diradical or cationic mechanisms, the structures of these two P450s provide insights into the binding of multiple substrates in one P450 active site and reveals important mechanistic features applicable to P450 intermolecular biaryl ring coupling enzymes.

4.3.3 Intramolecular biaryl ring coupling.
StaP catalyzes intramolecular C–C bond formation in the biosynthesis of staurosporine.[108,109] The crystal structure of StaP in complex with its substrate CPA (PDB ID: 2Z3U) revealed that the two carboxyl groups of CPA are bound in the active site *via* several hydrogen bonds and electrostatics interactions. The two indole rings are held in place by intramolecular T-shaped π–π interactions (Fig. 9C).[108] Combined with structural analysis, theoretical QM/MM calculations, along with mutagenesis studies, the catalytic mechanism of StaP was proposed.[109] The substrate CPA loses two protons *via* assistance of a Wat–His–Wat triad and subsequently transfers two electrons to Cpd I (Fig. 9C). The C–C bond formation was formed during the second electron transfer.[109] StaP dramatically lost enzymatic activity when incubated with a (C-11, C11′)-chloro derivative of CPA (CCA). The crystal structure of the StaP–CCA complex (PDB ID: 3A1L) revealed the absence of one of the water molecules in the Wat–His–Wat triad,[109] supporting the essential role of the Wat–His–Wat triad for the StaP-catalyzed coupling reaction. Furthermore, the activities of two StaP mutants, the His of the Wat–His–Wat
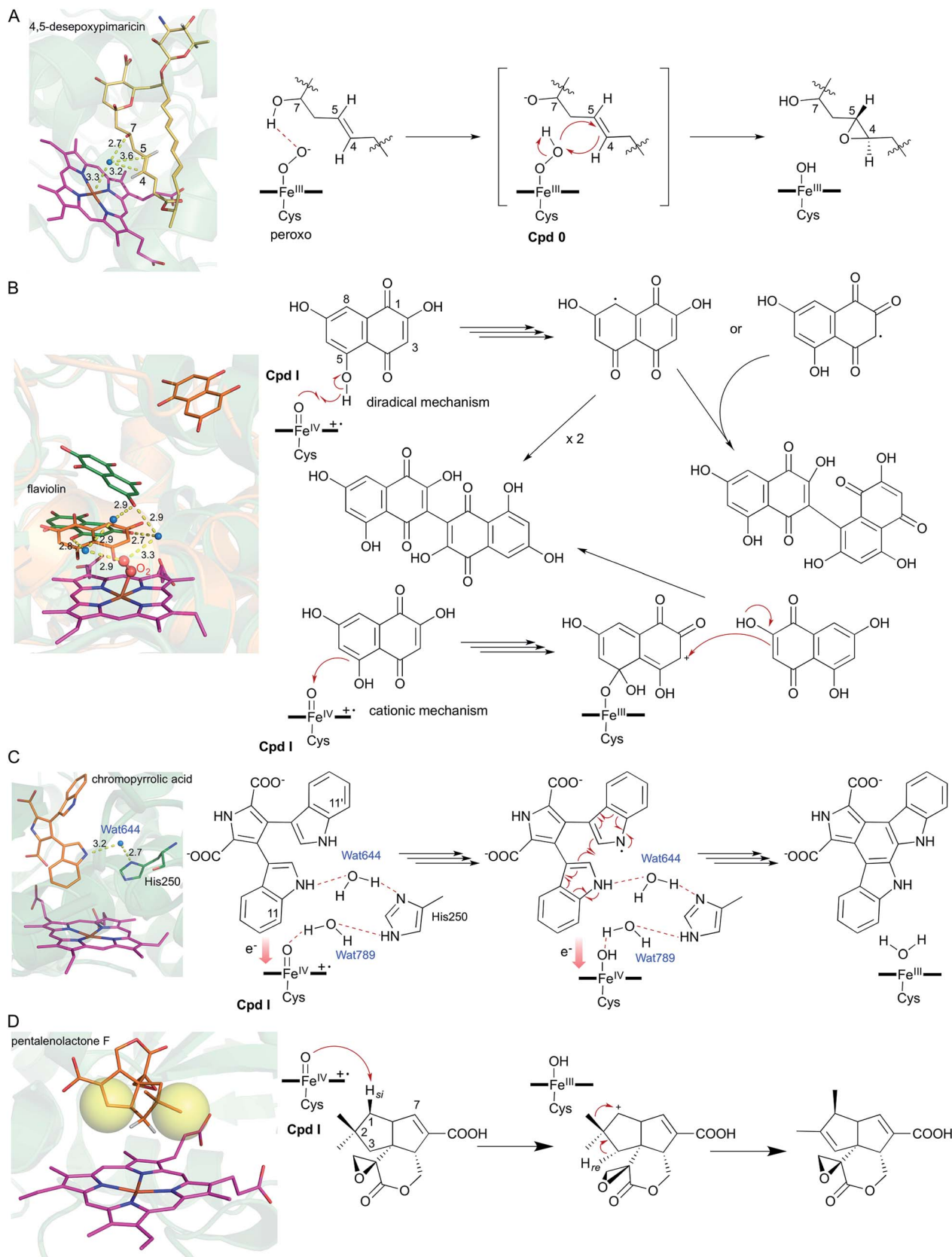
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Fig. 9 Structure-based mechanistic studies of P450s from *Streptomyces*. (A) Epoxidation by PimD *via* the hydroperoxyferric intermediate, Cpd 0 (PDB ID: 2XBK). (B) Substrate-assisted biaryl ring coupling by CYP158A1 and CYP158A2 (PDB IDs: 2NZ5 and 2D09). The two different binding modes of the two biflaviolin substrates are shown in orange and green. (C) Intramolecular biaryl ring coupling by StaP (PDB IDs: 2Z3U). Wat644 and His250 are shown in the active site; Wat789 is liberated during the formation of Cpd I (Wang 2009). (D) Oxidative rearrangement by PntM *via* a carbocation intermediate (PDB IDs: 5L1O). Blue dots in each figure represent water molecules; yellow spheres depict steric hindrance.

triad replaced by Ala and Phe, decreased to 25% and 72%, respectively, compared to that of wild-type StaP. These mutagenesis studies indicate that the His is not absolutely required for the proton relay pathway, and that a Wat–Wat diad, in absence of the His, may adequately relay protons for the StaP reaction.[109]

4.3.4 Oxidative rearrangement *via* a carbocation intermediate. PntM catalyzes a unique oxidative rearrangement in pentalenolactone biosynthesis.[113,114] The crystal structure of substrate-free PntM (PDB ID: 5L10) and in complex with various ligands (PDB ID: 5L1P, 5L1Q, 5L1R, 5L1S, 5L1T, 5L1U, 5L1V), including its substrate (pentalenolactone F), product (pentalenolactone), and substrate analogue (6,7-dihydropentalenolactone F), revealed that the three residues, Phe232, Met77, and Met81, which bind to the ligands, are unique in PntM and its orthologues.[114] The crystal structures support that C-1 of pentalenolactone F is sufficiently sterically hindered by its own axial $C-2_{si}$-methyl and the C-7-vinylidene substituents, that oxygen rebound does not occur after initial radical formation (Fig. 9D). Instead, electron transfer outcompetes the reduced oxygen rebound rate, resulting in carbocation formation at C-1. Subsequent C-2 methyl migration and C-3 deprotonation form pentalenolactone (Fig. 9D). The unusual carbocation intermediate is proposed to result from an outer shell electron transfer from the transiently generated C-1 radical to the heme-Fe^{III}–OH radical species.[114]

4.4 Structure-based engineering of P450s

P450s are well regarded as versatile biocatalysts that activate C–H bonds and perform a vast variety of chemistries. Crystal structures of P450s reveal insights into the shapes and sizes of active site cavities, how substrates bind and are oriented inside these typically hydrophobic pockets, and what controls regio- and stereoselectivity. Using details gleaned from these studies, structure-based enzymes or substrate engineering can be and have been carried out to rationally augment P450s as biocatalysts and generate novel natural product analogues.

4.4.1 Functional switch. AurH is a multifunctional enzyme involved in the biosynthesis of the polyketide antibiotic aureothin.[115–118] AurH catalyzes a tandem oxygenation of C-7 and C-9a resulting in tetrahydrofuran formation. The crystal structure of AurH is the first structure of the CYP151A subfamily (PDB ID: 3P3L, 3P3O, 3P3X, 3P3Z),[117] a relatively small group of P450s. A protein-ligand docking model revealed that the pyrone ring of the substrate occupies a hydrophobic pocket built by Phe85, Phe89, Leu175, Val242, Ala243, and Thr239.[117] The Phe89Trp and Thr239Phe mutants significantly decreased enzymatic performance and transformed the substrate into new aureothin derivatives (Fig. 10A).[117] The bulky Trp and Phe residues alter the binding mode of the pyrone entity *via* steric effects. Both engineered mutants catalyze the regioselective six-electron transfer of the nonactivated C-9a methyl group into a carboxylic acid *via* hydroxyl and aldehyde intermediates. New aureothin derivatives were isolated by fermentation of the *aurH*

(Phe89Trp)- and *aurH* (Thr239Phe)-complemented Δ*aurH* variant strains, respectively.[117] This work provides new insights into the finesse of enzyme-mediated oxygenation reactions, but also sets the stage for enzyme engineering in the field of synthetically useful biotransformations.

4.4.2 Regioselectivity switch. TxtE was the first enzyme reported to efficiently catalyze regioselective nitration of L-tryptophan.[122] The crystal structures of the substrate-free TxtE and in complex with L-tryptophan were reported (PDB ID: 4L36, 4TPN, 4TPO);[123,124] however, information about the interactions between L-tryptophan and the F–G loop was missing due to the disordered nature of the F–G loop. The F–G loop of TxtE was rebuilt *via* computing using Morkov state model molecular dynamics (MD) simulations, and three enzyme states, open lid, closed lid and transition, were proposed.[125] His176, from the F–G loop, plays a role in controlling the enzyme state (open, closed, and transition states) and forms edge-to-face π–π interactions with the substrate. Interestingly, His176Phe, His176Tyr, and His176Trp variants all resulted in nitration exclusively at the C-5 position instead of the C-4 position (Fig. 10B).[125] Computational models revealed that the increased steric demand of Phe/Tyr/Trp at position 176 shifts the substrate alignment relative to the heme group, placing the C-5 of indole closest to the nitrogen of the ferric peroxynitrite. This enzyme engineering shows a rare example of how a single residue in the P450 F–G loop can interact directly with the substrate to contribute to active-site organization and control regiochemistry.[125]

4.4.3 Substrate engineering. PikC performs multiple hydroxylation reactions on structurally diverse macrolides. The key to this significant substrate tolerance is the presence of the deoxyamino sugar desosamine.[186] The crystal structures of PikC in complex with its substrates, YC-17 and narbomycin (PDB ID: 2C6H, 2C7X), showed the desosamine moiety of the substrates is bound in two distinct binding pockets, one buried and the other surface-exposed.[186] Elimination of the surface-exposed negative charge at Asp50 results in a significant improvement in catalytic activity.[193] In the crystal structure of the PikC mutant Asp50Asn, the desosamine moiety of both YC-17 and narbomycin was bound in a catalytically productive "buried site" (PDB ID: 2WHW, 2WI9).[194] A two-step substrate binding mechanism was proposed with desosamine recognition in the two subsites to allow the macrolide substrate to sequentially progress toward a catalytically favorable orientation.[194] By harnessing its unique desosamine-anchoring functionality *via* a "substrate engineering" strategy, PikC is able to hydroxylate a series of carbocyclic rings linked to the desosamine glycoside *via* an acetal linkage in a regioselective manner.[195] Furthermore, the size, stereochemistry, and rigidity of the anchoring group influence the regioselectivity of enzymatic hydroxylation.[195] The natural anchoring group desosamine affords a 1 : 1 mixture of regioisomers, while synthetic anchors shift the ratio of C-10/C-12 hydroxylation of the YC-17 analogue from 1 : 4 to 20 : 1 (Fig. 10C).[195] Substrate engineering has provided new insights into the structural parameters that govern productive substrate binding and conversion of potential P450 substrates.
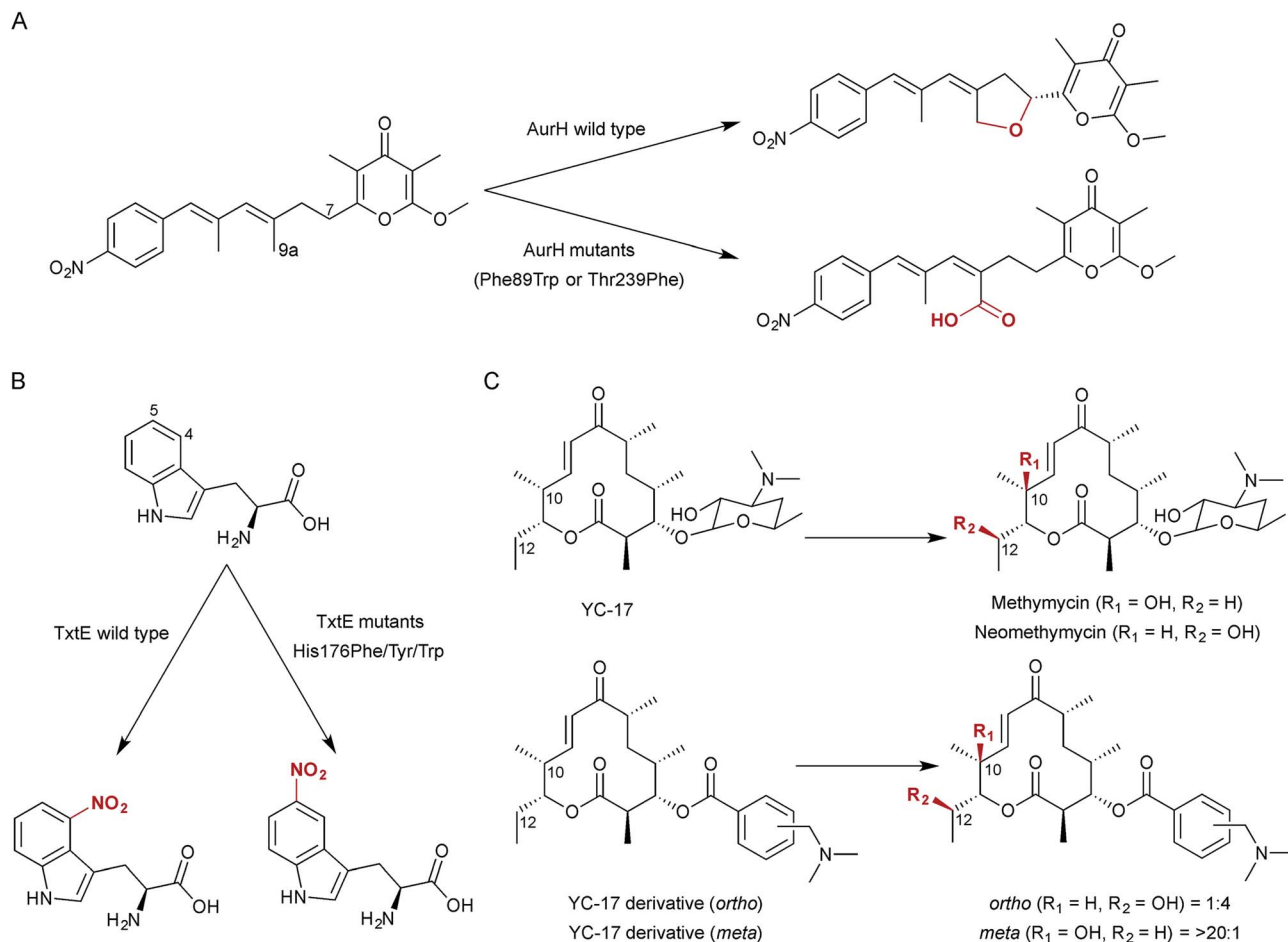
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A

B

C

Fig. 10 Structure-based engineering of P450s in *Streptomyces*. (A) Functional switch, from hydroxylation and ether formation to hydroxylation and oxidation to carboxylic acid, of AurH *via* site-directed mutagenesis. (B) Nitration regioselectivity switch, from C-4 to C-5, of TxtE *via* site-directed mutagenesis. (C) Substrate engineering of PikC shifting the natural abundance of regioisomers. The ratios representing the C-10 : C-12 hydroxylated products changes from 1 : 1 with the natural desosamine anchoring group to 1 : 4 or >20 : 1 depending on the synthetic anchoring variant used.

5 Conclusions and future perspectives

Genomics has changed the way scientists think about and study enzymes. Traditional enzymology begins with the observation of a phenomenon, and leads to the identification of the responsible protein followed by the encoding gene. With the advent and widespread use of DNA and whole genome sequencing, genes and their encoding proteins are known before any biochemistry is attempted. This leads to unlimited opportunities to discover new transformations, investigate enzyme mechanisms, and find patterns in sequence-structure-function relationships. However, with the rate of sequencing easily outpacing enzyme characterization, large-scale analysis and prioritization is needed to efficiently and effectively select the most promising candidates for further study. Analogous to strain prioritization for natural product discovery,[196–199] SSNs can be utilized to facilitate enzyme differentiation and prioritization.[161,200,201]

Cytochromes P450 are one of most exquisite and versatile biocatalysts found in nature. Although most of the research in P450 enzymology has focused on their direct relevance to human health through steroid biosynthesis and drug metabolism, P450s are key players in the biosynthesis of biologically active natural products. Due to their almost ubiquitous nature in secondary metabolite biosynthetic pathways, P450s possess tremendous diversity in sequence, substrate preference, and function. The P450s found within one of the most prolific producers of natural products, the *Streptomyces* genus (previously termed the CYPome[31]), and the SSNs described in this review, effectively confirms this premise. With only ~2.4% (even less with known physiological functions) and <0.4% of streptomycete P450s functionally and structurally characterized, respectively, it is clear that the true chemical and biological capabilities of these remarkable enzymes, in *Streptomyces* and all organisms, is not fully understood.

It is universally accepted that protein functions are directly related to their primary amino acid sequences and structures. The diversity and versatility, both in function and substrate

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preference, of P450s makes it extremely difficult to find a generalized rule-of-thumb for sequence–structure–function relationships. While the community does not currently have the capacity to correctly predict function or structure solely based on P450 sequence, efforts are continuing to understand these connections. Structural-based engineering and directed evolution of P450 enzymes for biotechnology applications have succeeded, but only on a case-by-case basis. Expanding the reaction space of microbial P450s, either through engineering or natural P450 variant discovery, will continue to improve the understanding and predictive power of sequence–structure–function relationships of P450s.

Natural P450 variants possess an immense capacity for chemical reactions and will dramatically advance the field of P450 enzymology, chemistry, and biotechnological applications. The discovery of natural TxtE variants that selectivity nitrate the C-5 position of tryptophan, after engineering a TxtE mutant to have the exact same regioselectivity,[125] perfectly highlights this idea. Nature, with its use of evolution and consequential diversity, has already developed many of the reactions and selectivities that scientists desire, and even ones that are still unimaginable. P450s are already used as an alternative to synthetic approaches to C–H functionalization. Discovery of new P450s, repurposing of known P450s, and taking inspiration from the natural product biosynthetic strategy of late-stage functionalization using regio- and stereoselective P450s will advance the field of biocatalysis and become a valuable technology.

Nature possesses the greatest diversity of chemical space. Enzymes are the natural catalysts that both biosynthesize and modify these complex scaffolds. New enzyme functions and selectivities found within natural product biosynthesis translate into novel natural products with unique chemical and biological characteristics. The prevalence of P450s to functionalize natural products, combined with the total number of uncharacterized enzymes, reinforces the idea of the vastness of natural product diversity that has yet to be discovered. Given the striking diversity of sequence and function that are found in P450s, the tremendous amount of available genomics data, and a precedence of P450s to catalyze unique or multifunctional transformations in natural product biosynthetic pathways, study of streptomycete P450s will undoubtedly continue to make fundamental contributions to enzymology, structural biology, natural products chemistry and biosynthesis.

6 Conflict of interest

There are no conflicts of interest to declare.

7 Acknowledgements

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