Streptomyces Cytochrome P450 Enzymes and Their Roles in the Biosynthesis of Macrolide Therapeutic Agents

Myung-A Cho, Songhee Han, Young-Ran Lim, Vitchan Kim, Harim Kim and Donghak Kim*

Department of Biological Sciences, Konkuk University, Seoul 05025, Republic of Korea

Abstract

The study of the genus Streptomyces is of particular interest because it produces a wide array of clinically important bioactive molecules. The genomic sequencing of many Streptomyces species has revealed unusually large numbers of cytochrome P450 genes, which are involved in the biosynthesis of secondary metabolites. Many macrolide biosynthetic pathways are catalyzed by a series of enzymes in gene clusters including polyketide and non-ribosomal peptide synthesis. In general, Streptomyces P450 enzymes accelerate the final, post-polyketide synthesis steps to enhance the structural architecture of macrolide chemistry. In this review, we discuss the major Streptomyces P450 enzymes research focused on the biosynthetic processing of macrolide therapeutic agents, with an emphasis on their biochemical mechanisms and structural insights.

Key Words: Streptomyces, P450, CYP, Biosynthesis, Macrolide, Secondary metabolite

INTRODUCTION

The phylum actinobacteria is one of the major lineages currently recognized within bacteria (Ventura et al., 2007). Actinobacteria are widely distributed in terrestrial, especially soil, and aquatic ecosystems (McCarthy and Williams, 1992; Stach and Bull, 2005). They are important for soil; they use extracellular enzymes to decompose and recycle complex mixtures of polymers in dead plant, animal and fungal materials (McCarthy and Williams, 1992). They also have diverse physiological and metabolic properties, including the ability to produce volatile substrates and secondary metabolites (Gust et al., 2003; Berdy, 2005).

Streptomyces is the largest genus among actinobacteria and a genus of the Streptomycetaceae family (Dyson, 2011). Filamentous Streptomyces is characterized by a complex life cycle; its morphological differentiation begins as a spore that germinates to produce a substrate (Chater and Chandra, 2006). Actinobacteria produce more than 10,000 bioactive compounds, of which the Streptomyces species produces approximately three-quarters (Berdy, 2005). Many of these bioactive compounds are potent antibiotic, antifungal, antitumor, immunosuppressant, antiviral, and antiparasitic agents. These properties have practical and important applications in clinical, veterinary, and agricultural fields. Soil-microorganisms became important to human health with the discovery of penicillin in 1928 by Fleming, and the discovery of the antituberculosis agent streptomycin from Streptomyces griseus in 1944 by Waksman (Ikeda, 2017). More recently, the 2015 Nobel prize in Physiology or Medicine was awarded to Omura and Campbell for their contributions to the discovery of the antiparasitic agent avermectin from Streptomyces avermitilis (Ikeda, 2017).

STREPTOMYCES P450

The cytochrome P450 enzymes (P450, CYP) are monooxygenases that catalyze a variety of metabolic and biosynthetic chemical reactions. These enzymes are of particular interest to the toxicology, drug metabolism, and pharmacology fields (Guengerich, 2001; Ortiz de Montellano, 2005). They exist throughout various forms of life, including animals, fungi, bacteria, and plants. In many organisms, the progressive completion of genome-sequencing projects has identified more than 15,000 P450 enzyme genes. These are systemically registered in the database (http://drnelson.uthsc.edu/CytochromeP450.html/). The Streptomycetaceae and Mycobacteriaceae genomes are interesting in that they carry an unusually large numbers of P450 genes; they are presumably...
involved in the biosynthesis of secondary metabolites. The fully sequenced genomes of *Streptomyces* species show that *S. coelicolor* A3(2) has 18 P450 genes, *S. avermitilis*, 33; *S. scabies*, 25; *S. peucetius*, 21; *S. hygroscopicus*, 7; and *Saccharopolyspora erythraea*, 22 within their chromosome sequences (Table 1) (Lamb et al., 2012).

### Table 1. *Streptomyces* cytochrome P450 enzymes and their putative reactions

| Organisms                  | P450 names | Putative reactions | References                        |
|----------------------------|------------|--------------------|------------------------------------|
| **Streptomyces coelicolor**| CYP105N1   | Hydroxylation (coelibilactin) | Lim et al., 2012                   |
|                            | CYP158A1   | Baryl ring coupling (C-C, flavilin) | Zhao et al., 2005, 2007            |
|                            | CYP158A2   | Baryl ring coupling (C-C, flavilin) | Zhao et al., 2005, 2007            |
|                            | CYP154C1   | Hydroxylation (Y-C-17, Pirkromycin, Narbomycin) | Podust et al., 2003               |
|                            | CYP154A1   | Unknown            | Lamb et al., 2002; Podust et al., 2004 |
|                            | CYP170A1   | Hydroxylation      | Zhao et al., 2009; Moody et al., 2012 |
|                            | CYP105D5   | Hydroxylation      | Chun et al., 2006, 2007            |
| **Streptomyces avermitilis**| CYP171A1   | Furan ring formation (Avermectin) | Ikeda and Omura, 1997             |
|                            | CYP107W1   | Hydroxylation (Oligomycin A) | Han et al., 2015, 2016            |
|                            | CYP107L2   | Hydroxylation (Y-C-17, Pirkromycin, Narbomycin) | Han et al., 2017                  |
|                            | CYP105P1   | Hydroxylation (Filipin) | Xu et al., 2009, 2010              |
|                            | CYP105D6   | Hydroxylation (Filipin) | Xu et al., 2009, 2010              |
|                            | CYP105D7   | Hydroxylation      | Pandey et al., 2010; Xu et al., 2015 |
|                            | CYP158A3   | Baryl ring coup (C-C) | Lim et al., 2017                   |
| **Streptomyces venezuelae** | CYP107L1   | Hydroxylation (Doxorubicin) | Xue et al., 1998a, 1999b; Dundas et al., 2006 |
| **Streptomyces rapamycinicus** | CYP112A2 | Oxidation (Rapamycin) | Graziani, 2009; Molnar et al., 1996 |
|                            | CYP107G1   | Hydroxylation (Rapamycin) | Graziani, 2009; Molnar et al., 1996 |
| **Streptomyces hygroscopicus** | CYP105U1  | Desaturation (Gelmanycin) | Rudolf et al., 2017               |
|                            | fkbD       | Hydroxylation (Ascomycin) | Wu et al., 2012                    |
| **Streptomyces peucetius**  | CYP129A2   | Hydroxylation (Doxorubicin) | Madduri and Hutchinson, 1995; Lomovskaya et al., 1999 |
|                            | CYP107P3   | Unknown            | Parajuli et al., 2004              |
|                            | CYP107U3   | Unknown            | Parajuli et al., 2004              |
|                            | CYP107N3   | Unknown            | Parajuli et al., 2004              |
|                            | CYP105F2   | Unknown            | Parajuli et al., 2004              |
|                            | CYP105P2   | Hydroxylation (Flavone) | Parajuli et al., 2004, 2007; Lee et al., 2016 |
| **Saccharopolyspora erythraea** | CYP147F1 | Hydroxylation (Fatty acids) | Bhattarai et al., 2013             |
|                            | CYP107A1   | Hydroxylation (Erythromycin A) | Shafiee and Hutchinson, 1987; Stassi et al., 1993 |
|                            | CYP113A1   | Hydroxylation (Erythromycin A) | Shafiee and Hutchinson, 1987; Stassi et al., 1993 |

*S. coelicolor* is a soil-dwelling, filamentous, gram-positive bacterium. It is the prototypic strain of the actinomycetes species (Lim et al., 2012). Therefore, its 8.7 Mb long chromosome sequence was completed early on; the results indicates a high (72.1%) GC content (Bentley et al., 2002). Also, it is predicted to contain 7,825 protein-encoding genes and 18 P450 enzyme genes (Lamb et al., 2002, 2010; Lim et al., 2012). Many of *S. coelicolor* P450 enzymes are involved in the biosynthesis of antibiotics and pigments. For example, CYP105N1 is an oxidase in the coelibilactin siderophore biosynthetic pathway. *S. coelicolor* has a cluster of genes predicted to synthesize a siderophore-related, non-ribosomal-encoded peptide designated as coelibilactin (Lim et al., 2012). The crystal structures of CYP105N1 were determined by the Waterman group and our group (Lim et al., 2012; Zhao et al., 2012). Both structures indicate that the wide-open conformation exposes the heme pocket and long I helix to the solvent (Fig. 1). This wide-open binding pocket is located above the heme group (Lim et al., 2012; Zhao et al., 2009; Moody et al., 2012). Further analyses indicate that the large open pocket to the active site is a key feature for easy accessibility to the peptidyl carrier protein-bound substrate, which performs many P450 catalytic reactions in a series of biosynthetic processes of Streptomyces (Lim et al., 2012).

The *S. coelicolor* genome contains two members of the CYP158A enzymes: CYP158A1, 2, and 3. CYP158A1 and CYP158A2 use oxidative C-C coupling in flavilin biosynthesis and their structures have been determined bound to flavi-
electron transport pathways of P450 enzymes in *S. coelicolor*. Deletion of the *sco3099* gene designated as *FDR* proteins coded by genes in *S. coelicolor* were constructed using PDB data (CYP105N1, 4FXB; CYP107W1, 4WQ0). The prosthetic heme is labeled in red and oligomycin is labeled in orange.

Fig. 1. Wide open conformation of the substrate binding pocket of CYP105N1 and CYP107W1. Electrostatic molecular surfaces of *Streptomyces* P450 enzymes are shown. 3D structural models were constructed using PDB data (CYP105N1, 4FXB; CYP107W1, 4WQ0). The prosthetic heme is labeled in red and oligomycin is labeled in orange.

Fig. 2. Chemical structures of metabolites synthesized by *Streptomyces*.

*Streptomyces avermitilis*

*S. avermitilis* has been named for its ability to produce avermectin, a 16-membered macrocyclic lactone derivative with potent anthelmintic and insecticidal properties. The 2015 Nobel Prize in Physiology or Medicine was awarded to Satoshi Omura and William C. Campbell for their contribution to the discovery of avermectin (http://www.nobelprize.org/). The 9-Mbp *S. avermitilis* genome contains approximately 7,600 open reading frames of proteins (Ikeda et al., 2003; Lamb et al., 2003) and 33 P450 genes, a relatively high number. These P450 genes are predicted to participate in the production of secondary metabolites, including antibiotics (Lamb et al., 2011).

The avermectin biosynthesis pathway contains three stages of processing: the formation of the polyketide-derived initial aglycon, the modification of the initial aglycon to generate avermectin aglycons, and glycosylation of avermectin aglycons to generate avermectins (Ikeda and Omura, 1997). The gene cluster of the second stage includes *aveE*, which encodes CYP171A1. CYP171A1 catalyzes the C6-C8a ring cyclase reaction of avermectin (Fig. 2) (Ikeda and Omura, 1997). Efforts to obtain recombinant CYP171A protein by our group and others have not been successful, therefore limited information is available about its biochemistry or catalytic properties.

Oligomycin A, an ATP synthase inhibitor is produced by the *olm* gene cluster of *S. avermitilis* (Fig. 2). The *olmB* gene encodes CYP107W1, which catalyzes the conversion of the macroclide oligomycin C (12-deoxy-oligomycin A) to oligomycin A through a hydroxylation step at C12. Our earlier study characterized the structure of the CYP107W1 and the P450 catalytic conversion of oligomycin C to oligomycin A (Han et al., 2015, 2016). Mass spectrometry analysis showed that CYP107W1 produced oligomycin A by regioselectively hydroxylation the C12 of oligomycin C (Han et al., 2015). Structural analysis of the CYP107W1-oligomycin A complex indicated that Trp178, located in the open pocket of the P450active site, is a critical residue that facilitates the productive binding conformation of large macrolide substrates (Fig. 1) (Han et al., 2016).

CYP107L2 is an orphan P450 of *S. avermitilis*, meaning its substrate or function is not identified. Its gene is located near a gene encoding a phenylacetate-degrading enzyme. CYP107L2 from *S. avermitilis* shares high sequence similarity with CYP107L1 (PikC) from *S. venezuelae*. Binding of pikromycin and lauric acid yielded the typical type I spectra, but no metabolic product was observed in the enzymatic reaction.
Streptomyces venezuelae

(Ban et al., 2017). Structural analysis of the CYP107L1-lauric acid complex showed that lauric acid is bound mainly by hydrophobic interactions to the carboxylate group of lauric acid. This indicates that significant conformational changes provide sufficient space for lauric acid in the substrate-binding site (Ban et al., 2017).

The Shou group characterized two P450 enzymes that catalyze the hydroxylation reaction of the polycyclon macrolide antibiotic filipin biosynthesis in S. avermitilis (Xu et al., 2009, 2010). This biosynthetic pathway contains two position-specific hydroxylations of filipin by the C26-specific CYP105P1 and C1-specific CYP105D6 (Fig. 2) (Xu et al., 2009). The crystal structure of the CYP105P1-filipin complex indicated that the distal pocket of CYP105P1 provides a specific environment for the large filipin substrate to bind with its pro-S side of position C26 directed toward the heme iron (Xu et al., 2010). A small sub-pocket in CYP105P1, but not in CYP105D6, differentiates the strict regiospecificity of these two P450 enzymes. Therefore, filipin cannot bind to CYP105D6 with the same orientation as in CYP105P1 due to steric hindrance (Xu et al., 2010). Furthermore, CYP105D7 from S. avermitilis was studied to determine its catalytic power in the metabolism of xenobiotic chemicals (Pandey et al., 2010; Xu et al., 2015). CYP105D7 catalyzed the 4'-hydroxylation reaction of the anti-inflammatory drug diclofenac and the 3'-hydroxylation reaction of isoflavone daidzein (Pandey et al., 2010; Xu et al., 2015). Interestingly, structural analysis showed that the active site pocket of CYP105D7 is relatively large and contains two diclofenac molecules, illustrating drug recognition with a double-ligand-binding mode (Xu et al., 2015).

One member of the biflavin synthase enzymes, CYP158A3, was found in S. avermitilis. Its amino acid sequence displays high similarity with CYP158A2, a biflavin synthase from S. coelicolor (Lim et al., 2017). A flavilox analog displayed similar type I binding upon titration with purified CYP158A3, indicating a P450 enzymatic reaction that forms pigment product S. avermitilis (Lim et al., 2017).

Streptomyces venezuelae

S. venezuelae is widely recognized for making chloramphenicol, the first antibiotic manufactured synthetically in large-scale. Yet, it also produces secondary metabolites including jadomycin and pikromycin (Bradley and Ritzi, 1968; Jakeman et al., 2009). Analysis of the full, approximately 9 Mb genomic sequence of Streptomyces venezuelae indicated 71% GC content and 8,080 protein coding genes (Song et al., 2016). Like other Streptomyces, some of its genome remains clustered among proteins involved in secondary metabolite biosynthesis pathways. The pik cluster includes genes which have roles for the biosynthesis of macrolide antibiotics such as narbomycin, pikromycin, methymycin and neomethymycin (Xue et al., 1998b). CYP107L1 (also called PikL) is found in the pik gene cluster and is a unique P450 in Streptomyces in that it can produce a number of related macrolide products from the gene cluster pathway (Xue et al., 1998a; Dyson, 2011). Interestingly, PikL can catalyze P450 reactions at different positions of macrolactones, using the 12-membered ring YC17 or the 14-membered ring narbomycin as substrates (Sharma et al., 2006; Dyson, 2011). Crystal structure analysis revealed a prominent feature of the PikL-macrolactone substrate interaction, which is the anchoring of the desosamine residue in two alternative binding pockets. This was based on a series of distinct amino acid residues that form a salt bridge and a hydrogen-bonding network with the deoxysugar C3’ dimethylamino group (Sherman et al., 2006).

Streptomyces rapamycinicus

The S. rapamycinicus strain was first isolated from soil on the Easter island and was earlier classed as Streptomyces hygroscopicus (Kumar and Goodfellow, 2008; Baranasic et al., 2013). This strain is the only organism that produces rapamycin, a macrolide immunosuppressant agent (Vezina et al., 1975; Alayev and Holz, 2013). Rapamycin is used to prevent organ transplant rejection, especially useful in kidney transplants, and used to coat coronary stents (Vezina et al., 1975). The immunosuppressive function of rapamycin is accomplished by inhibiting T- and B-cell activation by inhibiting mTOR to reduce their sensitivity to interleukin-2 (IL-2) (Mukherjee and Mukherjee, 2009).

In 2013, the Petkovic group obtained a draft genome sequence of the approximately 12.7 Mb S. rapamycinicus NRRL 5491 (Baranasic et al., 2013). This finding makes it possible to predict that S. rapamycinicus contains 10,425 protein-coding genes and 25 modular secondary metabolite clusters (Baranasic et al., 2013).

Since rapamycin is widely used in the medical field, the biosynthesis of rapamycin in this organism has been an area of intense focus. The gene cluster in rapamycin biosynthesis includes a type I polyketide synthase in conjunction with a non-ribosomal peptide synthetase often found in other macrolide biosynthesis pathways. Two P450 enzymes are involved in the rapamycin biosynthesis pathway, CYP112A2 (RepJ) and CYP107G1 (RepN) participate in the final steps of post-PKS tailoring steps, including oxidation and O-methylation reactions (Molnar et al., 1996; Graziani, 2009). Furthermore, they catalyze hydroxylation reactions at the C9 and C27 of pre-rapamycin macrolide, respectively (Fig. 2) (Chung et al., 2001). Recently, our group successfully obtained the recombinant proteins of these enzymes and their crystal structures have been analyzed (unpublished).

Streptomyces hygroscopicus

S. hygroscopicus has several subspecies and their classification is based on genomic similarity, however it is still difficult to determine features of its genome (Kumar and Goodfellow, 2010). In 2012, genomic analysis of Streptomyces hygroscopicus 5008 found that its 10.4 Mb genome has seven genes encoding P450 enzymes (Wu et al., 2012). Like other Streptomyces, S. hygroscopicus produces valuable secondary metabolites. Geldanamycin is an antitumor agent, which was discovered in S. hygroscopicus 17997 (He et al., 2006). Geldanamycin is a macrocyclic polyketide that is synthesized by a Type I polyketide synthase and a series of post-PKS tailoring steps including hydroxylation, O-methylation, carbamoylation, and oxidation (Lee et al., 2006). CYP105U1 (gdmP) is a P450 enzyme that participates in the geldanamycin biosynthesis pathway where it catalyzes the desaturation reaction (Rudolf et al., 2017). In addition, the FK520 gene cluster of S. hygroscopicus var. ascomyceticus (ATCC 14891) includes the fkbD gene encoding a P450 hydroxylase involved in ascomycin (FK520) biosynthesis. Ascomycin is an ethyl analog of tacrolimus (FK506) with strong immunosuppressant properties (Wu et al., 2012). However, there are no reports on the biochemical study of cytochrome P450 enzyme from S. hygroscopicus.
**Streptomyces peucetius**

*S. peucetius* strain produces the widely used daunorubicin and doxorubicin, which are clinically important anthracycline chemotherapeutic agents of the polyketide class of antibiotics (Lomovskaya *et al*., 1999). Its 8.7 Mb genome has 7,178 protein coding genes with 21 P450 enzymes including CY-P129A2 (DoxA) (Parajuli *et al*., 2004). The DoxA gene is found in the DNR-DXR gene cluster and its gene product, the enzyme CYP129A2, participates in the final hydroxylation step of the doxorubicin production process (Madduri and Hutchinson, 1995; Lomovskaya *et al*., 1999). Recently, the Oh group successfully purified the CYP129A2 enzyme and used mass spectrometric analysis to characterize its hydroxylation of resveratrol (Rimal *et al*., 2018).

**Saccharopolyspora erythraea**

*S. erythraea* produces the macrolide antibiotic erythromycin A. Full genomic sequencing revealed *S. erythraea* is an 8.2 Mb long, circular chromosome with 7,198 protein coding genes and at least 25 gene clusters for the biosynthesis of secondary metabolites (Oliynyk *et al*., 2007). Two P450s, CYP107P3, CYP107U3, CYP107N3, CYP105F2, and CYP105P2, are similar to the P450 enzymes of other organisms, but their functions are unclear (Parajuli *et al*., 2004). Interestingly, recombinant CYP105P2 showed flavone hydroxylase activity when assisted by the redox partner genes camA and camB (Niraula *et al*., 2012). The crystal structure of CYP105P2 indicates it has structural flexibility that can accommodate a broad range of ligands (Lee *et al*., 2016). CYP147F1 has ω-hydroxylation activity on long-chain fatty acids, and is therefore responsible for physiological processes in *S. peucetius* instead of producing secondary metabolites (Bhattarai *et al*., 2013).

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