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More P450s Are Involved in Secondary Metabolite Biosynthesis in *Streptomyces* Compared to *Bacillus*, *Cyanobacteria*, and *Mycobacterium*

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Abstract: Unraveling the role of cytochrome P450 monooxygenases (CYPs/P450s), heme-thiolate proteins present in living and non-living entities, in secondary metabolite synthesis is gaining momentum. In this direction, in this study, we analyzed the genomes of 203 *Streptomyces* species for P450s and unraveled their association with secondary metabolism. Our analyses revealed the presence of 5460 P450s, grouped into 253 families and 698 subfamilies. The CYP107 family was found to be conserved and highly populated in *Streptomyces* and *Bacillus* species, indicating its key role in the synthesis of secondary metabolites. *Streptomyces* species had a higher number of P450s than *Bacillus* and cyanobacterial species. The average number of secondary metabolite biosynthetic gene clusters (BGCs) and the number of P450s located in BGCs were higher in *Streptomyces* species than in *Bacillus*, mycobacterial, and cyanobacterial species, corroborating the superior capacity of *Streptomyces* species for generating diverse secondary metabolites. Functional analysis via data mining confirmed that many *Streptomyces* P450s are involved in the biosynthesis of secondary metabolites. This study was the first of its kind to conduct a comparative analysis of P450s in such a large number (203) of *Streptomyces* species, revealing the P450s’ association with secondary metabolite synthesis in *Streptomyces* species. Future studies should include the selection of *Streptomyces* species with a higher number of P450s and BGCs and explore the biotechnological value of secondary metabolites they produce.

Keywords: *Streptomyces*; *Mycobacterium*; *Bacillus*; *Cyanobacteria*; cytochrome P450 monooxygenases; secondary metabolites; biosynthetic gene clusters; terpenes; polyketides; P450 blooming; non-ribosomal peptides

1. Introduction

Cytochrome P450 monooxygenases (CYPs/P450s) are biotechnologically valuable enzymes [1]. P450s have heme (protoporphyrin IX), an iron(III)-containing porphyrin, as a prosthetic group in their
Because of the presence of this prosthetic group, these enzymes absorb wavelengths at 450 nm; thus, the name P450s has been assigned to these proteins. Since their identification, a large number of P450s have been identified in almost all living organisms and, surprisingly, in non-living entities as well. The regio- and stereo-specific catalytic nature of these enzymes makes them essential for the survival of some organisms, and these enzymes are thus good drug targets in the case of pathogenic organisms. Application of these enzymes in all fields of research continues, and excellent success has been achieved in using them for the production of substances valuable to humans or as drug targets or in drug metabolism, as reported previously. One of the applications of P450s currently being explored is their role in the production of secondary metabolites, compounds with potential biotechnological value, owing to their stereo- and regio-specific enzymatic activity, which contributes to the diversity of secondary metabolites.

Unlike other enzymes, P450 enzymes have a typical nomenclature system established by the International P450 Nomenclature Committee. According to the committee’s rules, P450s begin with the prefix “CYP” for cytochrome P450 monooxygenase, followed by an Arabic numeral which designates the family, a capital letter designating the subfamily, and an Arabic numeral designating the individual P450 in a family. The annotation of P450s (assigning family and subfamily) follows a rule that all P450s with >40% identity belong to the same family and all P450s with >55% identity belong to the same subfamily. Worldwide, researchers follow this P450 nomenclature system. The nomenclature of P450s is also be verified by phylogenetic analysis to enable their correct annotation, as phylogenetic-based annotation could detect similarity cues beyond a simple percentage identity cutoff, as mentioned elsewhere.

The continued genomic rush has resulted in genome sequencing of a large number of species belonging to all biological kingdoms. P450s in the newly sequenced species need to be annotated as per the International P450 Nomenclature Committee rules to enable researchers to use the same names for functional and evolutionary analysis of P450s. For this reason, large numbers of P450s have recently been annotated in bacterial species belonging to the genera Mycobacterium, Bacillus, Streptomyces, and Cyanobacteria. These studies have revealed numerous P450s involved in the synthesis of different types of secondary metabolites. This type of in silico study is highly important for identifying unique P450s that can be drug-targeted and for P450 evolutionary analysis, as the P450 profiles in species have been found to be characteristic of species’ lifestyle.

Among bacterial species, Streptomyces species are well-known for producing over two-thirds of the clinically useful antibiotics in the world. Because of this importance, Streptomyces species have been subjected to exhaustive secondary metabolite production studies. Streptomyces P450s play a key role in the production of different secondary metabolites; their contribution to secondary metabolite diversity and applications in drug metabolism have been reviewed extensively. In the latest study, comprehensive comparative analysis of P450 and secondary metabolite biosynthetic gene clusters (BGCs) in 48 Streptomyces species was elucidated. The study revealed the presence of novel P450s in Streptomyces species and numerous P450s forming parts of secondary metabolite BGCs. The study results indicated that lifestyle or ecological niches play a key role in the evolution of P450 profiles in species belonging to the genera Streptomyces and Mycobacterium.

To date, a large number of Streptomyces species genomes have been sequenced and are available for public use. This provided an opportunity to annotate P450s in these species to analyze and compare their profiles among different bacterial species, including the identification and comparative analysis of P450s involved in the production of secondary metabolites. This study thus aimed to perform genome data mining, annotation, and phylogenetic analysis of P450s in 155 newly available Streptomyces species genomes. It also included the identification and comparative analysis of P450s that are parts of secondary metabolite BGCs among bacterial species belonging to the genera Streptomyces, Bacillus, Mycobacterium, and Cyanobacteria, as the species belonging to these genera are known to have P450s and to produce secondary metabolites.
2. Results and Discussion

2.1. Streptomyces Species Have Large Number of P450s

Genome-wide data mining and annotation of P450s in 203 *Streptomyces* species (Supplementary Table S1) revealed the presence of 5460 P450s in their genomes (Figure 1, Table 1, and Supplementary Dataset 1). The P450 count in the *Streptomyces* species ranged from 10 to 69 P450s, with an average of 27 P450s. Apart from the complete P450 sequences, pseudo-P450s (6 hit proteins), P450-fragments (114 hit proteins), P450-derived glycosyltransferase activator proteins (22 hit proteins), and P450 false-positive hits (2 hit proteins) were also found in some *Streptomyces* species (Supplementary Table S2). The presence of these types of P450 hit proteins in species is common and, because of the nature of these proteins, they were not included in the study for further analysis. Among *Streptomyces* species, *Streptomyces albulus* ZPM was found to have the highest number of P450s in its genome (69 P450s) followed by *S. clavuligerus* (65 P450s); the lowest number of P450s was found in *Streptomyces* sp. CNT372 and *S. somaliensis* DSM 40738 (10 P450s each) (Figure 1 and Table 1). Analysis of the most prevalent number of P450s revealed that 19 P450s was the prevalent number in *Streptomyces* species (Table 1). The average number of P450s in *Streptomyces* species was found to be higher than in *Bacillus* species [22] and cyanobacterial species [23], and almost the same as in mycobacterial species [21] (Table 2). A point to be noted is that the number of species greatly influences the average number of P450s and, thus, the higher the number of species in the analysis, the better and more accurate the results, as mentioned elsewhere [20,23]. This is the reason *Streptomyces* species showed a slightly lower average number of P450s in their genomes compared to mycobacterial species, since only 60 species were employed in the study [21]. Thus, future annotation of P450s in more mycobacterial species will provide accurate insights into this aspect.

![Figure 1. Phylogenetic analysis of *Streptomyces* P450s. In total, 5460 P450s were used to construct the tree and the dominant P450 families are highlighted in different colors and indicated in the figure. A high-resolution phylogenetic tree is provided in Supplementary Dataset 2.](image-url)
Table 1. Genome-wide data mining and annotation of P450s in 203 Streptomyces species.

| Species Name | P450s No. | F No. | SF No. | Species Name | P450s No. | F No. | SF No. |
|--------------|-----------|-------|--------|--------------|-----------|-------|--------|
| Streptomyces sp. Ta6071 | 22 | 13 | 20 | Streptomyces sp. CTNT172 | 10 | 8 | 10 |
| Streptomyces purpurascens K2A81, ATCC 21405 | 22 | 17 | 20 | Streptomyces sp. CN5606 | 16 | 9 | 14 |
| Streptomyces sp. W007 | 28 | 12 | 24 | Streptomyces sp. SK30MFCA852 | 23 | 14 | 22 |
| Streptomyces sp. TA1486-18 | 18 | 12 | 17 | Streptomyces acidocaldarius 84-104 | 47 | 22 | 44 |
| Streptomyces lysofilicus ATCC 31396 | 25 | 19 | 24 | Streptomyces noursei NRRL 11379 | 19 | 10 | 16 |
| Streptomyces sp. PVA 94-07 | 20 | 7 | 18 | Streptomyces sp. OsgMP-M45 | 19 | 9 | 19 |
| Streptomyces sp. SPB78 | 20 | 12 | 20 | Streptomyces sp. AmelKG-A3 | 19 | 9 | 19 |
| Streptomyces canus 299MFChir4.1 | 28 | 17 | 27 | Streptomyces sp. S4 | 19 | 9 | 19 |
| Streptomyces sp. Psana1 | 30 | 15 | 29 | Streptomyces sp. SM8 | 18 | 8 | 16 |
| Streptomyces sulphuricus DSM 40104 | 26 | 13 | 25 | Streptomyces sp. LaPpAH-199 | 26 | 11 | 21 |
| Streptomyces sp. MopMF-M3 | 44 | 20 | 41 | Streptomyces sp. 14Co2L1E | 22 | 9 | 17 |
| Streptomyces coelicoflavus JA20606 | 17 | 12 | 16 | Streptomyces sp. DcaAA-21 | 24 | 10 | 22 |
| Streptomyces pristinaepratilis ATCC 25486 | 18 | 11 | 17 | Streptomyces sp. CTNT171 | 17 | 13 | 17 |
| Streptomyces sp. LaPpAH-201 | 19 | 8 | 19 | Streptomyces somaliensis DSM 40178 | 10 | 8 | 9 |
| Streptomyces albus CCRC. 11814 | 64 | 26 | 50 | Streptomyces sp. 351MFTu51 | 22 | 11 | 22 |
| Streptomyces viridochromogenes DSM 40736 | 24 | 15 | 24 | Streptomyces sp. DcaAA-83 | 24 | 10 | 22 |
| Streptomyces sp. LaPpAH-95 | 24 | 9 | 22 | Streptomyces sp. AmelKG-F2B | 24 | 17 | 23 |
| Streptomyces mirabilis YR139 | 42 | 26 | 41 | Streptomyces sp. CTNT02 | 26 | 13 | 22 |
| Streptomyces sp. A1529 | 26 | 15 | 24 | Streptomyces clausii DSM 40736 | 26 | 14 | 22 |
| Streptomyces sp. PVA 94-10 | 36 | 21 | 32 | Streptomyces sp. AA0539 | 19 | 10 | 19 |
| Streptomyces sp. CTNT18 | 27 | 15 | 24 | Streptomyces sp. OK008 | 31 | 13 | 27 |
| Streptomyces sp. CNH099 | 16 | 12 | 16 | Streptomyces sp. CN535 | 16 | 13 | 17 |
| Streptomyces sp. CNH287 | 16 | 12 | 16 | Streptomyces sp. Psana1 | 27 | 15 | 24 |
| Streptomyces sp. MnaMP-M77 | 32 | 14 | 27 | Streptomyces sp. WMMP322 | 19 | 11 | 17 |
| Streptomyces zincirestrictus K42 | 19 | 11 | 18 | Streptomyces sp. TOR520 | 20 | 13 | 19 |
| Streptomyces sp. Nl26MPSois12h | 22 | 11 | 19 | Streptomyces sp. AmelKG-E11A | 24 | 15 | 22 |
| Streptomyces sp. GXT6 | 13 | 8 | 11 | Streptomyces sp. PP42 | 16 | 6 | 14 |
| Streptomyces noursei NRRL 13998 | 19 | 10 | 16 | Streptomyces sp. DpondAA-E10 | 25 | 10 | 22 |
| Streptomyces sp. LaPpAH-108 | 24 | 12 | 23 | Streptomyces sp. HP8047 | 32 | 18 | 32 |
| Streptomyces aureofaciens JA 4570 | 30 | 20 | 30 | Streptomyces sp. DpondAA-A50 | 25 | 10 | 22 |
| Streptomyces hygrocarpicus ATCC 53653 | 57 | 21 | 49 | Streptomyces sp. TAA040 | 15 | 10 | 15 |
| Streptomyces sp. Tu 6176 | 30 | 15 | 26 | Streptomyces sp. Psana7 | 23 | 10 | 20 |
| Streptomyces ghaniensis ATCC 14672 | 35 | 20 | 34 | Streptomyces sp. PsanaD5 | 15 | 11 | 15 |
| Streptomyces sp. KICrAH-337 | 26 | 12 | 22 | Streptomyces sp. LamerL3-316 | 25 | 11 | 22 |
| Streptomyces sp. LaPpAH-202 | 19 | 8 | 19 | Streptomyces viridochromogenes TU57 | 31 | 17 | 29 |
| Streptomyces sp. UNC401CLCol | 15 | 11 | 15 | Streptomyces sp. GBA 94-10 | 20 | 7 | 18 |
| Streptomyces sp. SrixAA-H | 21 | 12 | 20 | Streptomyces sp. CNQ5-25 | 18 | 14 | 18 |
| Streptomyces lugdunensis Car8 | 28 | 20 | 27 | Streptomyces sp. SceaMF-e76 | 41 | 18 | 36 |
| Streptomyces sp. KICrAH-40 | 26 | 12 | 22 | Streptomyces mirabilis OK461 | 37 | 16 | 31 |
| Streptomyces roseosporus ACBC 10970 | 54 | 30 | 52 | Streptomyces sp. LaPpAH-185 | 44 | 27 | 40 |
| Streptomyces ganciulicus BK5-13-15 | 18 | 11 | 17 | Streptomyces exfoliatus DSMZ 41693 | 26 | 16 | 24 |
| Streptomyces auratus AG80001 | 35 | 14 | 33 | Streptomyces sp. PsTaAH-137 | 29 | 16 | 28 |
| Kitaatosporia sp. SLWpMP552h | 35 | 15 | 24 | Streptomyces sp. Amel2x9 | 27 | 15 | 26 |
| Streptomyces sp. NTK 937 | 17 | 8 | 17 | Streptomyces sp. AmelKG-D3 | 22 | 11 | 19 |
| Streptomyces sp. ScaMF-e48 | 19 | 10 | 17 | Streptomyces prunicolor NRRL 13075 | 44 | 18 | 39 |
| Streptomyces sp. Hmic12 | 25 | 14 | 24 | Streptomyces sp. e14 | 28 | 13 | 25 |
| Streptomyces griseoannulatus M045 | 16 | 11 | 16 | Streptomyces sp. CNX435 | 12 | 9 | 12 |
| Streptomyces diffusus M77 | 28 | 17 | 29 | Streptomyces sp. HCCBI0435 | 17 | 10 | 14 |
| Streptomyces sulphuricus L188 | 19 | 11 | 19 | Streptomyces sp. JS01 | 24 | 11 | 19 |
| Streptomyces sp. KICrAH-340 | 26 | 12 | 22 | Streptomyces chartreusius NRRL 3882 | 29 | 19 | 26 |
| Streptomyces sp. C | 30 | 17 | 27 | Streptomyces sp. CTN128 | 19 | 9 | 19 |
| Streptomyces indicaurantius SP6C | 13 | 8 | 12 | Streptomyces sp. Amel2x2 | 27 | 13 | 25 |
| Streptomyces sp. HGB020 | 23 | 13 | 22 | Streptomyces sp. LaPpAH-165 | 24 | 9 | 22 |
| Streptomyces sp. CNS615 | 27 | 15 | 24 | Streptomyces albus ZFM | 68 | 27 | 51 |
| Species Name | P450s | No. F | No. SF | Species Name | P450s | No. F | No. SF |
|--------------|-------|-------|--------|--------------|-------|-------|--------|
| *Streptomyces tsukubaensis* NRRL 18488 | 30 | 18 | 30 | *Streptomyces albus* NK660 | 64 | 27 | 50 |
| *Streptomyces venezuelensis* DSM 41686 | 18 | 10 | 15 | *Streptomyces noursei* | 64 | 26 | 52 |
| *Streptomyces* sp. SA2 AcG | 21 | 12 | 20 | *Streptomyces violaceoruber* Tu4113 | 31 | 19 | 30 |
| *Streptomyces bottropensis* ATCC 25435 (2517572239) | 31 | 19 | 30 | *Streptomyces* sp. | 49 | 26 | 44 |
| *Streptomyces albulus* NK660 | 64 | 27 | 50 | *Streptomyces rigaudonii* Tu4113 | 16 | 13 | 16 |
| *Streptomyces vitaminophilus* DSM 41686 | 18 | 10 | 15 | *Streptomyces sp. sp. 769* | 19 | 13 | 18 |
| *Streptomyces noursei* | 64 | 26 | 52 | *Streptomyces sp. CNQ360* | 16 | 13 | 16 |
| *Streptomyces sp. SA3 actG* | 21 | 12 | 20 | *Streptomyces violaceoruber* Tu4113 | 31 | 19 | 30 |
| *Streptomyces violaceoruber* Tu4113 | 50 | 16 | 42 | *Streptomyces sp. CNQ329* | 13 | 10 | 13 |
| *Streptomyces bottropensis* ATCC 25435 (2517572239) | 31 | 19 | 30 | *Streptomyces violaceoruber* Tu4113 | 31 | 19 | 30 |
| *Streptomyces bottropensis* ATCC 25435 (2517572239) | 31 | 19 | 30 | *Streptomyces sp. 142MF Col3.1* | 27 | 14 | 24 |
| *Streptomyces bottropensis* ATCC 25435 (2517572239) | 31 | 19 | 30 | *Streptomyces cattleya* NRRL 8058 | 25 | 11 | 23 |
| *Streptomyces bottropensis* ATCC 25435 (2517572239) | 31 | 19 | 30 | *Streptomyces cattleya* NRRL 8058 = DSM 46488 | 41 | 21 | 38 |
| *Streptomyces bottropensis* ATCC 25435 (2517572239) | 31 | 19 | 30 | *Streptomyces cattleya* NRRL 8058 = DSM 46488 | 41 | 21 | 38 |
| *Streptomyces bottropensis* ATCC 25435 (2517572239) | 31 | 19 | 30 | *Streptomyces cattleya* NRRL 8058 = DSM 46488 | 41 | 21 | 38 |
| *Streptomyces bottropensis* ATCC 25435 (2517572239) | 31 | 19 | 30 | *Streptomyces cattleya* NRRL 8058 = DSM 46488 | 41 | 21 | 38 |
| *Streptomyces bottropensis* ATCC 25435 (2517572239) | 31 | 19 | 30 | *Streptomyces cattleya* NRRL 8058 = DSM 46488 | 41 | 21 | 38 |

Abbreviations: No. F: number of P450 families; No. SF: number of P450 subfamilies.
Table 2. Comparative analysis of key features of P450s in different bacterial species.

|                        | Streptomyces Species | Mycobacterial Species | Bacillus Species | Cyanobacterial Species |
|------------------------|-----------------------|-----------------------|------------------|------------------------|
| Total No. of species analyzed | 203                   | 1784                  | 507              | 114                    |
| No. of P450s           | 5460                  | 1984                  | 507              | 341                    |
| No. of families        | 253                   | 77                    | 13               | 36                     |
| No. of subfamilies     | 698                   | 132                   | 28               | 79                     |
| Dominant P450 family   | CYP107                | CYP125                | CYP107           | CYP110                 |
| Average no. of P450s   | 27                    | 30                    | 4                | 3                      |
| No. of BGCs *          | 4457                  | 898                   | 1096             | 770                    |
| Average no. of BGCs    | 31                    | 15                    | 9                | 7                      |
| No. of P450s part of BGCs | 1231                 | 204                   | 112              | 27                     |
| Percentage of P450s part of BGCs | 22                  | 11                    | 22               | 8                      |
| Reference              | This work             | [20,21]               | [22]             | [23]                   |

Abbreviations: BGC: biosynthetic gene cluster. Symbol: * 103 cyanobacterial species [23] and 144 Streptomyces species were used for BGC analysis.

2.2. CYP107 Family Was Found to Be Dominant and Conserved in 203 Streptomyces Species

Analysis of P450 families and subfamilies in 203 Streptomyces species revealed that 5460 P450s could be grouped into 253 P450 families and 698 P450 subfamilies (Table 2 and Supplementary Table S3). Among Streptomyces species, S. clavuligerus had the highest number of P450 families (30) and P450 subfamilies (58) in its genome (Table 1). Although S. rimosus ATCC 10970 had the same number of P450 families as S. clavuligerus, the number of subfamilies was the third highest (52 subfamilies) (Table 1). One interesting observation is that the species with the highest number of P450s did not have the highest number of P450 families, suggesting that some of the P450 families were populated (bloomed). Blooming of P450 families is common across species, and this phenomenon has been observed in different species belonging to different biological kingdoms [24,26,34–36]. Phylogenetic analysis revealed that some of the P450 families were scattered across the evolutionary tree (Figure 1). This phenomenon was also observed previously for Streptomyces species P450s, and it has been hypothesized that the phylogenetic-based annotation of P450s could be detecting similarity cues beyond a simple percentage identity cutoff [20]. Analysis of P450 families in the 155 Streptomyces species used in this study revealed the presence of 38 new P450 families, i.e., CYP1200A1, CYP1216A1, CYP1223A1, CYP1228A1, CYP1236A1, CYP1238A1, CYP1265A1, CYP1279A1, CYP1369A1, CYP1432A1, CYP1518A1, CYP1529A1, CYP1543A1, CYP1568A1, CYP159A1, CYP1607A1, CYP1658A1, CYP1759A1, CYP1810A1, CYP1832A1, CYP1866A1, CYP1896A1, CYP1920A1, CYP1929A1, CYP1931A1, CYP1940A1, CYP1941A1, CYP1943A1, CYP1972A1, CYP1984A1, CYP1994A1, CYP2076A1, CYP2080A1, CYP2134A1, CYP2180A1, CYP2349A1, CYP2427A1, and CYP2723A1. A detailed analysis of the number of new P450 families found in different Streptomyces species is presented in Supplementary Table S2.

Among the P450 families, the CYP107 family was found to be dominant, with 1235 P450s in Streptomyces species, followed by CYP105 with 684 P450s, CYP157 with 525 P450s, and CYP154 with 510 P450s (Figure 2 and Supplementary Table S3), indicating the possible blooming of these families in Streptomyces species, as observed in species belonging to different biological kingdoms [24,26,34–36]. It is interesting to note that the CYP107 family was also found to be dominant in the Bacillus species [22], indicating its dominant role in the synthesis of secondary metabolites in both the Streptomyces and Bacillus genera. An interesting pattern was observed when comparing subfamily diversity in the dominant P450 families (Figure 2, Table 3, and Supplementary Table S3). P450 families such as CYP107, CYP105, CYP183, and CYP113 had the highest diversity at the subfamily level, as numerous subfamilies were found in these families (Supplementary Table S3). This phenomenon of the highest diversity in P450 families being found in Streptomyces species is not uncommon, and this proved to be the key contributor in the production of diverse secondary metabolites in Streptomyces species compared to mycobacterial species [20]. Strong support for this argument is the fact that the CYP105 P450 family members in Streptomyces species have been shown to be involved in oxidation of numerous
endogenous and exogenous compounds and in the generation of different secondary metabolites [32]. However, in contrast to the diversity at subfamily level for the P450 families CYP107, CYP105, CYP183, and CYP113, the rest of the dominant P450 families had single or double or triple subfamilies, indicating subfamily-level blooming in these P450 families (Table 3).

Figure 2. P450 family and subfamily analysis in 203 Streptomyces species. Only the dominant P450 families with more than 40 P450s are shown in the figure. Detailed data on P450 families and subfamilies are presented in Supplementary Table S3.

Table 3. P450 subfamily analysis in the dominant families in 203 Streptomyces species. The number of members in the dominant P450 subfamily is presented. Detailed data on different subfamilies are presented in Supplementary Table S3.
P450 family conservation analysis revealed that the CYP107 family is conserved in all 203 *Streptomyces* species (Figure 3 and Supplementary Dataset 3). P450 families such as CYP156, CYP105, CYP154 and CYP157 are also present in the majority of the *Streptomyces* species (Figure 3 and Supplementary Dataset 3).

![Heat-map of P450 family conservation analysis in *Streptomyces* species.](image)

**Figure 3.** Heat-map of P450 family conservation analysis in *Streptomyces* species. In the heat-map, the presence and absence of P450 families are indicated in red and green colors. The horizontal axis represents P450 families and the vertical axis represents *Streptomyces* species.

2.3. Numerous P450s Involved in Secondary Metabolite Production in *Streptomyces* Compared to Other Bacterial Species

Analysis of 144 *Streptomyces* species’ genomes revealed the presence of 4457 BGCs in their genomes (Table 2 and Supplementary Table S4). The number of BGCs found in 144 *Streptomyces* species was found to be higher than in mycobacterial, *Bacillus*, and cyanobacterial species (Table 2), indicating the superiority of the *Streptomyces* species in producing secondary metabolites; two-thirds of the antibiotics used in the world currently come from these species [28]. The average number of BGCs in *Streptomyces* species was found to be double compared to mycobacterial species and close to four times higher than that in *Bacillus* and cyanobacterial species (Table 2). Analysis of BGCs revealed that a large proportion of *Streptomyces* species’ P450s are part of BGCs compared to other bacterial species; 1231 P450s in *Streptomyces* species compared to 112 in *Bacillus* species, 204 in mycobacterial species, and 27 in cyanobacterial species (Table 2). A total of 1231 P450s were found to be part of BGCs belonging to 135 P450 families (Figure 4 and Supplementary Table S5). Among 135 P450 families, P450s belonging
to the CYP107 family were dominantly present in BGCs, followed by CYP105, CYP157, and CYP154 (Figure 4 and Supplementary Table S5). This clearly suggests that the P450 families that are bloomed in *Streptomyces* species are actually involved in the production of secondary metabolites. This strongly supports the proposed hypothesis that in *Streptomyces* species, P450s are evolved to generate secondary metabolites, thus helping these bacteria to thrive in their environment [20]. In order to assess the *in silico* results generated by this study, in which a large number of *Streptomyces* species P450s were predicted to be involved in secondary metabolite production, we performed an extensive literature review to identify *Streptomyces* P450s involved in the production of secondary metabolites. As shown in Table 4, a large number of P450s belonging to different P450 families, as predicted in this study, were found to be involved in the production of different secondary metabolites. This strongly supports the notion that the P450s identified as part of different BGCs in this study produce secondary metabolites.

Figure 4. Analysis of P450s associated with secondary metabolite production in *Streptomyces* species. (A) Dominant P450 families (families with higher numbers of members) that are part of biosynthetic gene clusters (BGCs) and (B) dominant BGCs (present in higher numbers) containing P450s were presented in the figure. The numbers next to bars indicate the number of P450s in panel A and the number of BGCs in panel B. Detailed information is presented in Supplementary Table S5.

Analysis of P450 BGCs revealed the presence of 235 types of BGCs, where the BGC type, such as terpene, was dominant, followed by T1PKS, NRPS, and T3PKS (Figure 4 and Supplementary Table S5). A detailed analysis of P450s and types of BGCs containing P450s is presented in Supplementary Table S5. Analysis of the linkage between a particular P450 family and BGC revealed that some P450s are linked to a particular BGC (Supplementary Table S4), indicating horizontal transfer of BGCs among different organisms is well-documented in the literature [37].

Table 4. List of *Streptomyces* species P450s involved in synthesis of secondary metabolites.

| P450     | Species                  | Function                              | References       |
|----------|--------------------------|---------------------------------------|------------------|
| CYP158A1 | *Streptomyces coelicolor* A3(2) | Flavilin biosynthesis                 | [38]             |
| CYP1048A1| *Streptomyces scabiei*    | Thaxtomin (phytotoxin) biosynthesis   | [39]             |
| CYP105A1 | *Streptomyces griseolus*  | Diterpenoids synthesis                | [40]             |
| CYP105A3 (P450sca-2) | *Streptomyces carboxylinus* | Pravastatin synthesis                | [41]             |
| CYP105B28(GfsF) * | *Streptomyces graminifaciens* | Macrolide antibiotic synthesis       | [42,43]          |
| CYP105D6 | *Streptomyces avermitilis* | Filipin biosynthesis                  | [44]             |
| CYP105D7 | *Streptomyces avermitilis* | Filipin biosynthesis                  | [45]             |
| CYP105D8 | *Streptomyces tuberculatus* strain 1-1529 | Avermectin oxidation               | [32,46]          |
Table 4. Cont.

| P450     | Species                  | Function                                           | References |
|----------|--------------------------|----------------------------------------------------|------------|
| CYP105D9 | Streptomyces sp. JP95    | Griseorhodin biosynthesis                          | [32,47]    |
| CYP105F2 | *Streptomyces peucetius* | Oleandomycin biosynthesis                          | [48,49]    |
| CYP105H1 | Streptomyces noursei ATCC 11455 | Nystatin biosynthesis                           | [32]       |
| CYP105H3 | *Streptomyces natalensis* | Pimaricin biosynthesis                              | [32,50]    |
| CYP105H4 (AmplN) |  |
| CYP105H5 | *Streptomyces griseus*   | Candidicin biosynthesis                             | [32]       |
| CYP105K1 | *Streptomyces tendae strain Tue901* | Nokkomycin biosynthesis                           | [32,53]    |
| CYP105K2 | *Streptomyces ansaehromogenes* | Nokkomycin biosynthesis                           | [32]       |
| CYP105L1 (TyH1orf7) |  | Tylosin biosynthesis                              | [54,55]    |
| CYP105L4 (ChmH1) |  | Chalcomycin biosynthesis                          | [36]       |
| CYP105M1 (orf10) |  | Clavulanic acid antibiotic biosynthesis            | [57]       |
| CYP105N1 | *Streptomyces coelicolor A3(2)* | Coelobactin siderophore biosynthesis               | [58,59]    |
| CYP105P1 | *Streptomyces avermitilis* | Filipin biosynthesis                                | [44]       |
| CYP105U1 | *Streptomyces sp. HK803* | Geldanamycin biosynthesis                          | [60]       |
| CYP105A1 | *Streptomyces tuberclidicus strain R922* | Avermectin oxidation                         | [32,46]    |
| CYP105A2 | *Streptomyces tuberclidicus strain 1-1529* | Avermectin oxidation                         | [32,46]    |
| CYP107A1 | *Streptomyces pescuetius* | Dealkylation of 7-ethoxycoumarin                  | [62]       |
| CYP107A1 | *Saccharopolyspora erythraea* | Erythromycin biosynthesis                         | [63,64]    |
| CYP107B (HmtN) |  | Himastatin biosynthesis                           | [65]       |
| CYP107B (HmtN) | *Streptomyces himastatinicus ATCC 53653* | Himastatin biosynthesis                           | [66]       |
| CYP107C1 | *Streptomyces thermotolerans* | Carbomycin biosynthesis                           | [67]       |
| CYP107E4 (chmPII) |  | Chalcomycin biosynthesis                          | [36]       |
| CYP107E2 (chmPII) |  | Chalcomycin biosynthesis                          | [36]       |
| CYP107F5 (Tam1) |  | Tiranadycin biosynthesis                          | [68,69]    |
| CYP107G1 | *Streptomyces rapamycinicus* | Rapamycin biosynthesis                            | [70,71]    |
| CYP107G1 (rapN) |  | Rapamycin biosynthesis                            | [71,72]    |
| CYP107G1 | *Saccharopolyspora erythraea* | Erythromycin biosynthesis                         | [63,64]    |
| CYP113B1 (TyH1) |  | Tylosin biosynthesis                              | [54,55]    |
| CYP113D3 (HmtT) |  | Himastatin biosynthesis                           | [65]       |
| CYP113D3 (HmtT) |  | Himastatin biosynthesis                           | [66]       |
| CYP113A1 | *Saccharopolyspora erythraea* | Erythromycin biosynthesis                         | [63,64]    |
| CYP113D3 (HmtT) |  | Himastatin biosynthesis                           | [65]       |
| CYP113H1 (HmtS) |  | Himastatin biosynthesis                           | [66]       |
| CYP112A2 | *Streptomyces rapamycinicus* | Rapamycin biosynthesis                            | [70,71]    |
| CYP112A3 | *Streptomyces hiroscopius* | Rapamycin biosynthesis                            | [70,71]    |
| CYP112A4 (FkbD) |  | Rapamycin biosynthesis                            | [70,71]    |
| CYP122A2 | *Streptomyces tsukubaisis* | FK506 (immunosuppressant) polyketide biosynthesis | [77]       |
| CYP129A2 | *Streptomyces peucetius* | Doxorubicin biosynthesis                           | [78,79]    |
| CYP129A2 (dox A) |  | Doxorubicin biosynthesis                           | [80,81]    |
| CYP131A2 (dntQ) |  | Doxorubicin biosynthesis                           | [80,81]    |
| CYP140M1 (Tnl) |  | Tautomyacin biosynthesis                          | [82]       |
| CYP151A (AurH) |  | Aureothin biosynthesis                            | [83]       |
| CYP154A1 | *Streptomyces coelicolor A3(2)* | Polyketide synthesis and cyclization of a cellular dipentaenone | [84,85]    |
Table 4. Cont.

| P450     | Species                        | Function                        | References   |
|----------|--------------------------------|---------------------------------|--------------|
| CYP154B1 | Streptomyces fradiae            | Tylosin biosynthesis            | [54,55]      |
| CYP154C1 | Streptomyces coelicolor A3(2)   | Macrolide biosynthesis          | [86]         |
| CYP158A2 | Streptomyces coelicolor A3(2)   | Flavonol biosynthesis           | [87]         |
| CYP161A2 | Streptomyces natalensis         | Pimaricin biosynthesis          | [88]         |
| CYP161A3 | Streptomyces nodous             | Amphoterin biosynthesis         | [51]         |
| CYP162A1 | Streptomyces tendae             | Nikkomycin biosynthesis         | [53,89]      |
| CYP163A1 | Streptomyces spheroidis         | Novobiocin biosynthesis         | [80]         |
| CYP163B3 | Streptomyces sp. Acta 2897      | Skyllamycin biosynthesis        | [91]         |
| CYP170A1 | Streptomyces coelicolor A3(2)   | Albaflavenone biosynthesis      | [92]         |
| CYP170A2 | Streptomyces avermitilis        | Albaflavenone biosynthesis      | [93]         |
| CYP170B1 | Streptomyces albus              | Albaflavenone biosynthesis      | [94]         |
| CYP171A1 | Streptomyces avermitilis        | Avermectin biosynthesis         | [85,96]      |
| CYP183A1 | Streptomyces avermitilis        | Pentalenolactone biosynthesis   | [96,97]      |
| CYP244A1 | Streptomyces sp tp-at0274       | Rapamycin biosynthesis          | [70,71]      |
| CYP245A1 | Streptomyces sp tp-at0274       | Rapamycin biosynthesis          | [70,71]      |
| CYP246A1 | Streptomyces scabiei            | Thaxtomin (phytotoxin) biosynthesis | [98]      |
| CYP248A1 | Streptomyces thiolatus           | Aureothin biosynthesis          | [83]         |

Note: For some P450s, protein notations are given in parentheses. These P450s were annotated in this study (indicated with asterisk superscript) and previously (indicated with exclamation mark) [20] by browsing the individual biosynthetic gene-cluster sequences reported in the literature. To enable readers to match the P450s with the published literature, we have provided protein notations in the parentheses. If known, the name of the secondary metabolite of which P450s are involved in production is indicated in the table.

3. Materials and Methods

3.1. Information on Streptomyces Species and Genome Database

In total, 203 Streptomyces species genomes (permanent and finished draft genomes) available for public use at the Joint Genome Institute Integrated Microbial Genomes and Microbiomes (JGI IMG/M) [99] and Kyoto Encyclopedia of Genes and Genomes (KEGG) [100] were used in this study. The 203 Streptomyces species included 48 Streptomyces species for which P450s and BGCs were annotated previously [20]. For these 48 species, P450 and BGCs data were retrieved from published articles and used in the study [20]. Thus, 155 Streptomyces species were data-mined for P450s and BGCs in this study. Information on the species used in the study is provided in Supplementary Table S1.

3.2. Genome Data Mining and Identification of P450s

Identification and annotation of P450s in Streptomyces species were carried out following a method described elsewhere [20–22]. Briefly, each Streptomyces species genome available at JGI IMG/M [99] was searched for P450s using the InterPro code “IPR001128”. The hit protein sequences were then searched for the presence of P450 characteristic motifs such as EXXR and CXG [101]. Proteins having one of these motifs were considered pseudo-P450s, and proteins that were short in amino acid length and lacking both motifs as P450 fragments. Neither the pseudo-P450s nor the P450 fragments were considered for further analysis.

3.3. Allocating Family and Subfamily to P450s

The hit proteins that were collected were subjected to BLAST analysis against bacterial P450s at the website http://www.p450.unizulu.ac.za/. Based on the International P450 Nomenclature Committee rule [17–19], proteins with a percentage identity greater than 40% were assigned to the same family as named homolog P450s, and those that had greater than 55% identity were assigned to the same
subfamily as named homolog P450s. Proteins that had a percentage identity less than 40% were assigned to a new family.

3.4. Streptomyces P450 Phylogenetic Analysis

Phylogenetic analysis of the Streptomyces P450s was carried out following the method described in the literature [102]. First, the Streptomyces P450 sequences were aligned using the MAFFT v6.864 program with an automatically optimized model option [103], available at the Trex web server [104]. The alignments were then automatically subjected to inference and optimization of the tree by the Trex web server with its embedded weighting procedure, and the best inferred tree was visualized and annotated by iTOL [105].

3.5. Streptomyces P450 Profile Heat-Maps

P450 profile heat-maps were generated following a method published previously [22,27] to check the presence and absence of P450s in Streptomyces species. Briefly, a tab-delimited file was imported into Multi-Experiment Viewer (Mev) [106] and hierarchical clustering using a Euclidean distance metric was used to cluster the data. In total, 203 Streptomyces species formed the vertical axis and P450 family numbers formed the horizontal axis. Data were presented as −3 for family absence (green) and 3 for family presence (red).

3.6. Identification of P450s That Are Part of Secondary Metabolite BGCs

Secondary metabolite BGCs analysis and identification of P450s that are part of these BGCs were carried out following the procedure mentioned previously [102], with slight modification. For each Streptomyces species genome available at JGI IMG/M, the secondary metabolite BGCs were searched for the presence of P450s. The DNA sequence of BGCs with P450s was collected and formatted to fasta format using PSPad editor (http://www.pspad.com/en/). The fasta-formatted files were then used to identify the type of cluster and most similar known clusters using the Antibiotics and Secondary Metabolite Analysis Shell (anti-SMASH) program [107]. The results obtained were recorded on Excel spreadsheets and represented as species-wise BGCs, type and similar known BGCs, percentage similarity to known BGCs, and P450s that are part of specific BGCs. Some Streptomyces species genome IDs did not pass through anti-SMASH analysis, and thus these species were not included in P450s analysis as part of secondary metabolite BGCs. A list of Streptomyces species subjected to anti-SMASH analysis is presented in Supplementary Table S4.

3.7. Data Analysis

All calculations were done following the method described in the literature [23]. The average number of P450s was calculated using the formula: Average number of P450s = Number of P450s/Number of species. The average number of BGCs was calculated using the formula: Average number of BGCs = Total number of BGCs/Number of species. The percentage of P450s that formed part of BGCs was calculated using the formula: Percentage of P450s part of BGCs = 100 × Number of P450s part of BGCs /Total number of P450s present in species. For comparative analysis of P450s and BGCs, information for bacterial species belonging to the genera Bacillus [22], Mycobacterium [21], and Cyanobacteria [23] was resourced from published articles.

4. Conclusions

In the last five decades, research on cytochrome P450 monooxygenases (CYPs/P450s) has mainly focused on their function and structural aspects, with little focus on evolutionary analysis, especially in microbes. The availability of a large number of microbial species genomes gives us an opportunity to focus on exploring the evolutionary aspects of P450s. Because a typical nomenclature system that has been established for P450s, each species genome needs to be data-mined and P450 proteins need
to be annotated (assigning family and subfamily). In this way, researchers around the world can make use of uniform P450 names. In this study, we therefore annotated a large number of P450s in 203 *Streptomyces* species and found 38 new P450 families. Some P450 families were found to be bloomed in *Streptomyces* species even at the subfamily level. Comparative analysis of key P450 features among different bacterial species revealed that *Streptomyces* species had a greater number of P450s, more secondary metabolite BGCs, and the highest number of P450s as part of BGCs compared to the bacterial species belonging to the genera *Bacillus*, *Mycobacterium*, and *Cyanobacteria*. This further confirmed that the higher the number of P450s, the higher the secondary metabolite diversity in a species. This was true for *Streptomyces* species, as large number of P450s were found to be involved in the generation of diverse secondary metabolites. One interesting phenomenon observed was the linkage between a particular P450 family and BGC. This indicates that these BGCs were horizontally transferred among different *Streptomyces* species. This study is a good addition to the comparative analysis of P450s and BGCs among different bacterial populations. Data presented in the study will serve as a reference for further annotation of P450s in *Streptomyces* species and other bacterial species. In *silico* predicted BGCs need to be experimentally validated to assess the secondary metabolites' biological properties.

**Supplementary Materials:** Supplementary materials can be found at http://www.mdpi.com/1422-0067/21/13/4814/s1.

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