Exploring the Electron Transfer Pathway in the Oxidation of Avermectin by CYP107Z13 in \textit{Streptomyces hygroscopicus} ZB01

Mei Li, Yujie Zhang, Lin Zhang, Xiaoyan Yang, Xiliang Jiang*

State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China

Abstract

\textit{Streptomyces hygroscopicus} ZB01 can effectively oxidize \textit{4'-OH} of avermectin to form \textit{4''-oxo-avermectin}, CYP107Z13 is responsible for this site-specific oxidation in ZB01. In the present study, we explored the electron transfer pathway in oxidation of avermectin by CYP107Z13 in ZB01. A putative [3Fe-4S] ferredoxin gene \textit{fd68} and two possible NADH-dependent ferredoxin reductase genes \textit{fdr18} and \textit{fdr28} were cloned from the genomic DNA of ZB01. \textit{fd68} gene disruption mutants showed no catalytic activity in oxidation of avermectin to form \textit{4''-oxo-avermectin}. To clarify whether \textit{FdR18} and \textit{FdR28} participate in the electron transfer during avermectin oxidation by CYP107Z13, two whole-cell biocatalytic systems were designed in E. coli BL21 (DE3), with one co-expressing CYP107Z13, \textit{Fd68} and \textit{FdR18} and the other co-expressing CYP107Z13, \textit{Fd68} and \textit{FdR28}. Both of the two biocatalytic systems were found to be able to mediate the oxidation of avermectin to form \textit{4''-oxo-avermectin}. Thus, we propose an electron transfer pathway NADH→\textit{FdR18/\textit{FdR28}}→\textit{Fd68}→CYP107Z13 for oxidation of avermectin to form \textit{4''-oxo-avermectin} in ZB01.

Introduction

\textit{Streptomyces} spp. produces many important natural products, including many known antibiotics. Cytochrome P450 enzymes (CYP450s) are involved in these biosynthetic and biotransformation reactions [1,2]. P450s are heme-dependent monoxygenases that catalyze the insertion of oxygen atoms from atmospheric oxygen molecules into carbon–hydrogen bonds within a diverse range of organic compounds [3]. Emanectin benzoate is a derivate of avermectin, a potent semisynthetic insecticide used to control many agriculturally important pests. Oxidation of \textit{4'-OH} into \textit{4''-oxo} of avermectin is one key reaction step in the synthesis of emanectin benzoate from avermectin [4]. Direct regiospecific chemical oxidation of the \textit{4'-OH} group in avermectin to form \textit{4''-oxo-avermectin} is precluded by the high reactivity of the 5-OH group in the molecule, necessitating a protection–deprotection strategy (Fig. 1). Avoiding these additional steps would greatly reduce the complexity of the production process along with the final cost of emanectin benzoate. CYP107Zs from \textit{Streptomyces} were reported to have the capability to oxidize \textit{4'-OH} into \textit{4''-oxo} of avermectin regioselectively [5].

Many CYP450s from bacterial were found to be class I type electron transfer systems. Including CYP113 family from gram-positive alkane-degrading bacteria [6,7], CYP199A4 from \textit{Rhodopseudomonas palustris} HaA2 [8], CYP105 family, CYP107 family and other CYPs from \textit{Streptomyces} [3,5,9,10]. Classical class I type electron transfer system, consists of an FAD-containing ferredoxin reductase (FdR), an iron–sulfur protein ferredoxin (Fd), and a cytochrome P450 (P450) [11]. Electrons are delivered from the reduced pyridine nucleotide coenzymes NAD(P)H to P450 via Fd and FdR [12], and the Fd are usually [2Fe-2S] type, although there are also reports on the use of ferredoxins of other cluster types. In \textit{S. griseus}, both a [3Fe-4S] ferredoxin and a 7 Fe ferredoxin, that contains a [3Fe-4S] as well as a [4Fe-4S] cluster, were shown to deliver electrons to CYP105D1 (P450soy) [3]. In \textit{Bacillus subtilis}, a [4Fe-4S] cluster ferredoxin was suggested as a potential redox partner of CYP107H (P450Biol) [13]. Only a few Fds and FdRs from bacterial P450 systems have been purified and characterized because of their instability and relatively low expression levels [14–16].

In our previous study, we screened an \textit{S. hygroscopicus} strain ZB01 which can oxidize \textit{4'-OH} of avermectin to form \textit{4''-oxo-avermectin} with greater efficiency than those of reported functional \textit{Streptomyces} [15,17]. CYP107Z13 was found to be responsible for this site-specific oxidation in ZB01 [18]. In this study, we explored the electron transfer process in the oxidation of avermectin by CYP107Z13 in ZB01. A ferredoxin gene \textit{fd68} and two ferredoxin reductase genes \textit{fdr18} and \textit{fdr28} were cloned from ZB01, and we found that there exist an electron transfer pathway NADH→\textit{FdR18/\textit{FdR28}}→\textit{Fd68}→CYP107Z13 in ZB01 for oxidation of avermectin to form \textit{4''-oxo-avermectin}.
Materials and Methods

Bacterial Strains and Plasmids

The microorganisms and plasmids used in this study are listed in Table 1. *S. ahygroscopicus* ZB01 (CGMCC No. 2804) was isolated and maintained in our laboratory, and was grown in liquid YEME medium or on YMS agar [19]. The protoplast regeneration medium was R2YE [19]. *E. coli* DH5α (Trans, Beijing) was used for bacterial transformation and plasmid propagation. *E. coli* BL21 (DE3) (Trans, Beijing) was used for recombinant protein expression and whole-cell biocatalytic systems. For the plasmid-containing cultures, 100 μg ml⁻¹ ampicillin and/or 50 μg ml⁻¹ kanamycin for *E. coli* strains or G418 (10 μg ml⁻¹ for *E. coli* strains and 5 μg ml⁻¹ for *Streptomyces* strains) instead of apramycin were added.

Cloning of Ferredoxin and Ferredoxin Reductase Genes

Genomic DNA of *S. ahygroscopicus* ZB01 was prepared according to Kieser et al. (2000) and used as the template for PCR reaction. Primers used for PCR are listed in Table 2. According to the conserved region of the flanking sequence of Fd genes from *Streptomyces* in NCBI, a pair of primers Fd1 and Rd1 were designed for cloning Fd gene in ZB01. PCR amplification was performed using 1 μM primers and LA Taq polymerase with GC buffer (TaKaRa, Japan). The PCR program was as follows: denaturation at 94°C for 4 min; followed by 30 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 2 min; and a final extension of 72°C for 10 min. For cloning FdR genes from ZB01, Primer pairs F1/R1 and F2/R2 were designed based on the known FdR genes in NCBI to amplify the full-length and partial FdR gene fragment respectively in ZB01 in combination with the La Taq DNA polymerase in GCI buffer (TaKaRa, Japan). The PCR program used was as follows: denaturation at 94°C for 5 min; 30 to 32 cycles of 94°C for 1 min, 63°C for 1 min, and 72°C for 1.5 min; and a final extension at 72°C for 10 min. The resulting 1.3-kb PCR product (fdr18 gene) using F1/R1 and the 0.6-kb PCR product (partial sequence of fdr28 gene) using F2/R2 were cloned into the pMD19-T vector system. The correct clones were

![Diagram of avermectin and product 4'-O-avermetacin](image)

**Figure 1. Structures of the avermectin and product 4'-O-avermetacin.**
doi:10.1371/journal.pone.0098916.g001

![Table 1](image)

**Table 1. Microorganisms and plasmids used in this study.**

| Strain or plasmid | Source |
|------------------|--------|
| *E. coli* DH5α   | Routine cloning host Beijing TransGen Biotech Co. Ltd. |
| *E. coli* BL21 (DE3) | T7 system expression host Beijing TransGen Biotech Co. Ltd. |
| *E. coli* z13     | *E. coli* BL21 (DE3) containing pRSET-z13 This study |
| *E. coli* fdr18 | *E. coli* BL21 (DE3) containing pRSET-fdr18 This study |
| *E. coli* fdr28 | *E. coli* BL21 (DE3) containing pRSET-fdr28 This study |
| *E. coli* zfr18  | *E. coli* BL21 (DE3) containing pRSET-z13 and pDuet-fd-fdr18 This study |
| *E. coli* zfr28  | *E. coli* BL21 (DE3) containing pRSET-z13 and pDuet-fd-fdr28 This study |
| *S. ahygroscopicus* ZB01 | CGMCC 2804, cyp107z13, fdr68, fdr18 and fdr28 producer This lab |
| ZBA/fdr68-3     | fdr68 disruption mutant of *S. ahygroscopicus* ZB01 This study |
| ZBA/fdr68-6     | fdr68 disruption mutant of *S. ahygroscopicus* ZB01 This study |
| pMD19-T Easy    | TA cloning vector, Amp<sup>B</sup> This study |
| pKC1139         | *Streptomyces-E. coli* conjugative shuttle vector, Am<sup>R</sup> Bierman et al. (1992) |
| pKC1139::fdr68  | 172 bp fragment of fdr68 into Hind III- and EcoR I-cut pKC1139 This study |
| pRSET-b         | Expression vector in *E. coli*, Amp<sup>B</sup> Novagen, |
| pRSET-fdr18     | pRSET-b carrying fdr18 This study |
| pRSET-fdr28     | pRSET-b carrying fdr28 This study |
| pRSET-z13       | pRSET-b carrying cyp107z13 This study |
| pRSFDuet-1      | Vector for co-expressing two proteins in *E. coli*, Km<sup>R</sup> Novagen |
| pRSFDuet-fd-fdr18 | pRSFDuet-1 carrying fdr68 and fdr18 This study |
| pRSFDuet-fd-fdr28 | pRSFDuet-1 carrying fdr68 and fdr28 This study |

doi:10.1371/journal.pone.0098916.t001
confirmed by sequencing. The full-length fdr28 gene sequence was cloned using a genome walking kit (TaKaRa, Japan). Three rounds of thermometric asymmetric nested PCR were performed using arbitrary primers (AP1–AP4) and specific primers (spF1-3 and spR1-3) were used to amplify the 3' and 5' flanking regions of the 0.6-kb known sequence, respectively. All the PCR products were cloned into the pMD18-T vector system and the correct clones were confirmed by sequencing. Plasmid manipulation, transformation of E. coli, restriction digestion, DNA fragment isolation and cloning techniques were performed according to standard procedure [19,20] and the manufacturer’s instructions.

**Disruption of Ferredoxin Gene**

Fd gene was inactivated by gene disruption via single-crossover recombination. A 172 bp fragment from fd68 named Δfd68 was amplified by PCR with primers Fd2/Rd2 and genomic DNA of S. ahygroscopicus ZB01 as the template. The PCR product was then subcloned into the HindIII/EcoRI digested pKC1139 [21] to generate the gene disruption vector pKC1139::fd68. pKC1139::fd68 was propagated in E. coli DH5α and transformed into S. ahygroscopicus ZB01 protoplasts mediated by PEG [22]. The transformants were selected for G418 resistance, and were then induced by a high temperature at 39°C for 48 h to obtain fd68 disruption mutant strains. The disruption strains were cultivated on YMS medium [19] for more than 3 generations without G418 selection for obtaining stable resistant transformants. Apramycin resistance gene and fd68 gene were analyzed by PCR for confirmation that fd68 disruption mutants have the correct structure. The colony morphologies were observed with the naked eye. Sporulation was checked using optical microscope. The growth rates of strains were measured by analysis of mycelium biomasses [19]. Each experiment was repeated for three times.

**Expression, Purification and Characterization of Ferredoxin Reductases**

For expression FdR proteins in E. coli, pRSET-fdr18 plasmid was constructed by inserting fdr18 gene into EcoRI and HindIII sites of pRSET-b. pRSET-fdr28 plasmid was constructed by

| Primer Name | Nucleotide sequences (5’–3’) | Enzyme site | Products |
|-------------|-----------------------------|-------------|----------|
| Fd1         | GATACCTCGGGATGATGAGC        | None        | fdr68    |
| Rd1         | ACACCGCGGAGTGAAGGCC         | None        | fdr18    |
| F1          | GTTGTCGACGCAACAGCG          | None        |         |
| R1          | CTACGGGAACGATGMSGCAG        | None        |         |
| F2          | GAAACCGCGGAGCTTCTCA         | None        |         |
| R2          | CAGAACACGCGGAGGTGCA         | None        | 0.6 kb fdr28 gene fragment |
| spF1        | ACTCGGAGGACATCCGCA          | None        |         |
| spF2        | GCCCGAAACGATCTACGGA         | None        |         |
| spF3        | GCCATCAACGCGAGTGCCT        | None        |         |
| spR1        | CGTGCAGATGATGAGCAACC       | None        |         |
| spR2        | TCGCTCGCAGTCAACTGACTA      | None        |         |
| spR3        | ACCGCTGACCCCTAGCTCA       | None        | full-length fdr28 gene |
| tF          | TAGAATTCATGCTGACGCAACAGCG  | EcoRI       |         |
| tR          | TTAAGCTTCTACGGGAGCACTGCACTGCAG | HindIII    | fdr18    |
| eF          | TAAGATCTATGGTCGAGGTGCTACT   | Bgl II      |         |
| eR          | TTAAGCTTCTACGGGAGCACTGCACTGCAG | HindIII   | fdr28    |
| Fd2         | ATTAAGCTGGCTGTATGCGTGTCGTCGT | HindIII    |         |
| Rd2         | ATAGAAATACCTCGAGAATCTCTGCTGA | EcoRI    | Δfd68    |
| AF1         | GCTCTACGGTGACCTTCACACCCTGG | None        |         |
| AR1         | CACCTCCTGCCCAAGGCAAGC      | None        | apramycin resistance gene |
| ff1         | ATGGCAGATCAACTCGGACACC    | None        |         |
| fR1         | TCGGCTCAGCCTGCTGCTGA      | None        | fdr68    |
| z13F        | GAGAGTCTATGACAGCAACTAAGGACTCCCC | Bgl II    |         |
| z13R        | CGGAGTCTATGACGCAAGGAGCTGACCGCAG | EcoRI | cyp107z13 |
| RfdF        | GAGAGTCTATGACGCAAGGAGCTGACCGCAG | Bgl II |         |
| RfdR        | GGGGTACCTGCAGTCTCTGCTGCTGCTGAGTGG | Kpn I | fdr68    |
| Rzre1F      | AAGAATTTATGTCGAGCAGCACCAGCG | EcoRI       |         |
| Rzre1R      | AAAGAATTTATGTCGAGCAGCACCAGCG | HindIII    | fdr18    |
| Rzre2F      | TAGAATTTATGTCGAGCAGCACCAGCG | EcoRI       |         |
| Rzre2R      | TTAAGCTTCTACGGGAGCTGTAAGGCAACCGCTGCTG | HindIII | fdr28   |

doi:10.1371/journal.pone.0098916.t002
inserting the fd28 gene into Bgl II and Hind III sites of pRSETb. Primers fR/fR and eF/eR were used for amplifying fd18 and fd28 genes respectively, where the native GTG start codons were changed into ATG (in bold) to facilitate the expression in E. coli (Table 2). E. coli BL21 (DE3) containing the expression constructs were grown in LB medium supplemented with 100 µg ml⁻¹ ampicillin at 37°C until OD₆₀₀ reached 0.6. Isopropyl-thio-b-D-galactopyranoside (IPTG) was used as the inducer and δ-aminolevulinic acid was used as the heme precursor at a final concentration of 0.5 mM. The strain was allowed to grow for 6 h at 28°C. The cells were used for extracting recombinant proteins. The recombinant proteins were purified through Ni-Sepharose 6 fast flow column (GE Healthcare) and eluted with elution buffer containing 100 mM δ-aminolevulinic acid, 0.5 mM IPTG and 100 µg ml⁻¹ ampicillin in isopropanol were added to the cultures. Further after incubation for 6 h at 28°C, the cultures were processed for detecting avermectin and its derivatives using HPLC [18].

Bioinformatic Analysis

Sequence similarity analysis and alignment were carried out using the BLASTX, DNAMan (5.0), and CLUSTAL X programs. The amino acid sequence was predicted using the SWISSPROT database via ExPaSy. The isolectric points and molecular weights of the predicted proteins were calculated using a PROTPARAM tool and DNAMan (5.0). The online tool SignalP 4.1 was used for signal peptide analysis.

Nucleotide Sequence Accession Numbers

The nucleotide sequences of ferredoxin gene fd68, ferredoxin reductase genes fd18 and fd28 reported in this paper were deposited into the GenBank database under accession numbers KC147630, KC147631 and KC510106, respectively.

Results

Cloning and Sequence Analysis of Ferredoxin Gene fd68,
Ferredoxin Reductase Genes fd18 and fd28

A 1810 bp DNA fragment was cloned from S. ahygroscopicus ZB01 genome by PCR with primers F1/R1, and an open reading frame of 195 bp within the fragment was obtained and named fd68. The GC content of fd68 was as high as 71.3%. The deduced Fd68 contains 63 amino acids with a molecular weight of 7.1 kDa and was a putative iron-sulfur protein (3Fe-4S) Fd, with the conserved iron-sulfur cluster binding sites coordinated with 3Fe-4S type iron sulfur cluster [25] at Cys10, 16 and 54 (Fig. 2A). Fd68 exhibits 89.1% identity and 88.9% similarity to Fd232 of S. tubercidicus I-529 and Fd232 of S. tubercidicus R-922 [5].

FdR gene from ZB01 DNA was amplified by PCR using primers F1/R1 and was named fd18. A 600 bp fragment was amplified using primers F2/R2, and the upstream and downstream flanking sequence regions were obtained by three rounds of nested PCR. The combined full-length FdR gene was named fd28.

For detection of the whole-cell biocatalytic activities of E. coli BL21(DE3), co-expressing CYP107Z13, Fd and FdR, the strains were cultured in 3 ml LB with 100 µg ml⁻¹ ampicillin and 30 µg ml⁻¹ kanamycin at 37°C for 8 h and then transferred into 30 ml fresh LB, supplemented with appropriate antibiotics, and cells were allowed to grow at 37°C for 2 h. And then 0.5 mM δ-aminolevulinic acid (ALA), 0.5 mM IPTG and 100 µg ml⁻¹ avermectin in isopropanol were added to the cultures. After further incubation for 6 h at 28°C, the cultures were processed for detecting avermectin and its derivatives using HPLC [18].

Electron Transfer Pathway of CYP107Z13 Oxidation Avermectin

For detection of the whole-cell biocatalytic activities of E. coli BL21(DE3), co-expressing CYP107Z13, Fd and FdR, the strains were cultured in 3 ml LB with 100 µg ml⁻¹ ampicillin and 30 µg ml⁻¹ kanamycin at 37°C for 8 h and then transferred into 30 ml fresh LB, supplemented with appropriate antibiotics, and cells were allowed to grow at 37°C for 2 h. And then 0.5 mM δ-aminolevulinic acid (ALA), 0.5 mM IPTG and 100 µg ml⁻¹ avermectin in isopropanol were added to the cultures. After further incubation for 6 h at 28°C, the cultures were processed for detecting avermectin and its derivatives using HPLC [18].

Biological Characteristics of fd68 Disruption Mutants

To elucidicate the function of fd68 gene, a fd68 gene disruption vector pKC1139::fd68 was constructed (Fig. 3A) and transformed into ZB01. Two stable G418 resistant transformants ZBΔfd68-3

Avermectin Catalytic Activity Detection

Spores of S. ahygroscopicus ZB01 and fd68 disruption mutant strains were grown in ISP-2 liquid medium at 30°C with shaking at 200 rpm for two days. 100 mg avermectin ml⁻¹ in isopropanol was then added and the spores were cultured for another two days. Avermectin and their derivatives were extracted with methyl-t-butyl ether, collected and redissolved in acetonitrile, and were finally detected using HPLC [18].
and ZBΔfδ68-6 were selected. The plasmid, pKC1139: fδ68 could not be extracted from these two mutants (data not shown), so fδ68 and apramycin resistance genes were analyzed by PCR using the genomic DNA of the mutants as templates. There was an intact fδ68 gene (about 200 bp) and no apramycin resistance gene in wild S. ahygroscopicus ZB01, while no intact fδ68 gene was amplified at the presence of apramycin gene fragments (about 500 bp) in ZBΔfδ68-3 and ZBΔfδ68-6 (Fig. 3B), suggesting that the fδ68 gene had been integrated into the chromosome of ZB01 and disruption had occurred in ZBΔfδ68-3 and ZBΔfδ68-6.

The colony morphologies of wild S. ahygroscopicus ZB01, ZBΔfδ68-3 and ZBΔfδ68-6 on YMS agar were similar during the first four days, but the ZB01 colonies started to turn gray gradually from the fifth day and most colonies were gray till 7 days, while the ZBΔfδ68-3 and ZBΔfδ68-6 colonies were still white at the seventh days (Fig. 3C). ZB01 and the two mutant strains produced similar amounts of spores as observed under an optical microscope, but the two mutant strains showed a 36–48% decrease in mean biomasses from the fourth to the sixth day. (Fig. 3D).

HPLC analysis of the metabolites of avermectin was presented in Fig. 5D. UV-visible spectra analysis demonstrated that absorption peaks appeared at 388, 453, and 482 nm for oxidized FdR18 and at 386, 455, and 486 nm for FdR28 (Fig. 4C). The electron transport rates of FdR18 and FdR28 for NADH and NADPH, showing that both of the proteins are possible FAD-dependent FdRs (Fig. 4D). The molecular weight of FdR28 was greater than FdR18 on SDS-PAGE (Fig. 4B). The expressed target proteins from E. coli into E. coli BL21(DE3) and transformant E. coli BL21 (DE3) were co-transformed into E. coli BL21 (DE3) and the resultant transformant E. coli-zfr18 and pDuet-zfr18-28 were co-transformed into E. coli BL21 (DE3) and transformant E. coli-zfr28 were obtained. Both of E. coli-zfr28 and E. coli-zfr28 showed cytochrome c oxidase activities of E. coli-zfr18 and E. coli-zfr28 were analyzed by SDS-PAGE (Fig. 5C). The expressed target proteins from E. coli-zfr18 and E. coli-zfr28 were co-transformed into E. coli BL21 (DE3) and transformant E. coli-zfr28 were analyzed by SDS-PAGE (Fig. 5C).

Whole-cell Biocatalytic Systems for Oxidation of Avermectin Using E. Coli

For co-expressing CYP107Z13, Fd68 and FdR28 in E. coli, pRSET-z13, pDuet-fd-fdr18 and pDuet-fd-fdr28 were constructed (Fig. 5A). pRSET-z13 and pDuet-fd-fdr18 were co-transformed into E. coli BL21 (DE3) and the resultant transformant E. coli-zfr18, pRSET-z13 and pDuet-fd-fdr18 were co-transformed into E. coli BL21 (DE3) and transformant E. coli-zfr28 were obtained. Both of E. coli-zfr28 and E. coli-zfr28 showed cytochrome c oxidase activities of E. coli-zfr18 and E. coli-zfr28 were analyzed by SDS-PAGE (Fig. 5C).

HPLC analysis of the metabolites of avermectin was presented in Fig. 5D. The expressed target proteins from E. coli-zfr18 and E. coli-zfr28 were analyzed by SDS-PAGE (Fig. 5C). The electron transport rates of FdR18 and FdR28 for NADH and NADPH were detected using DCPIP as the electron acceptor. The Km and Kcat of FdR18 for NADH, evaluated using DCPIP, was 64 μM and 121 min⁻¹, whereas those of FdR28 for NADH were 25.4 μM and 386 min⁻¹, respectively. Both FdR18 and FdR28 proteins showed higher electron transport activity against NADH than NADPH, showing that both of the proteins are possible NADH-dependent FdRs (Fig. 4D).

Whole-cell Biocatalytic Systems for Oxidation of Avermectin Using E. Coli

For co-expressing CYP107Z13, Fd68 and FdR28 in E. coli, pRSET-z13, pDuet-fd-fdr18 and pDuet-fd-fdr28 were constructed (Fig. 5A). pRSET-z13 and pDuet-fd-fdr18 were co-transformed into E. coli BL21 (DE3) and the resultant transformant E. coli-zfr18, pRSET-z13 and pDuet-fd-fdr18 were co-transformed into E. coli BL21 (DE3) and transformant E. coli-zfr28 were obtained. Both of E. coli-zfr28 and E. coli-zfr28 showed cytochrome c oxidase activities of E. coli-zfr18 and E. coli-zfr28 were analyzed by SDS-PAGE (Fig. 5C).
ically oxidize avermectin to 4′-oxo-avermectin (Fig. 5D). Conversion efficiency was found to be 16% in wild type ZB01 and zero in negative control strains, while it was 11.2% and 0.6% in E. coli-zfr28 and E. coli-zfr18 respectively. These results showed that both FdR18 and FdR28 sustained the oxidization activity of avermectin to form 4′-O-avermectin, with electron transfer efficiency of FdR28 to Fd68 higher than that of FdR18 to Fd68.

Discussion

The electron transfer of P450s from Streptomyces are of the classical class I system, which constitutes ferredoxin reductase (FdR), ferredoxin (Fd), and P450. Electrons are delivered from reduced pyridine nucleotide coenzymes NAD(P)H to P450 via an FdR with flavin adenine dinucleotide and an iron–sulfur protein Fd [26]. There are many CYP450 genes and relatively few Fd and FdR genes in Streptomyces. Nineteen CYP450s, two Fd genes and four FdR genes were found in S. Peuculate genome [27], eighteen CYP450s, six Fd and four FdR genes were found in S. coelicolor A3(2) genome, and thirty-three CYP450s, nine Fd and six FdR genes were found in S. avermitilis genome [28]. CYP450 of Streptomyces has a high specificity to Fd and FdR as the electron transfer proteins for special catalytic function. CYP105A1 can only accept electrons transported from Fd1 and CYP105B1 can only accept electrons from Fd2 to perform the 7-ethoxycoumarin hydroxylation reaction in S. griseolus [10]. The electron transport pathway of hydroxylation fatty acid CYP105D5 in S. coelicolor A3(2) was NADH → FdR1 → Fd1 → CYP105D5 [26]. In our previous studies, we found that S. ahgycoplicus ZB01 had a strong catalytic activity for the region-specific oxidation of 4′-OH of avermectin to form 4′-oxo-avermectin. Resting S. ahgycoplicus ZB01 cells can convert 36% of the avermectin substrate to 4′-oxo-avermectin in 72 h at avermectin concentrations of 1 g l⁻¹ measured by HPLC [17], whereas the resting S. tuberculosis I-1529 cells can convert 16% of the avermectin substrate to 4′-oxo-avermectin in 96 h at avermectin concentrations of 0.75 g l⁻¹ which was reported to be the highest biocatalytic activity reported by Molnar [5]. CYP107Z13 was responsible for this regio-specific
oxidation of avermectin. In this study, we cloned one Fd gene \( fd \), two FdR genes \( fdr \) and \( fdr \), and found that there exist the electron transfer pathway NADH \( \rightarrow \text{FdR18/FdR28} \rightarrow \text{Fd68} \rightarrow \text{CYP107Z13} \) in ZB01 in oxidation of avermectin to form 4\(^{-}\)-oxo-avermectin.

Many studies on \( \text{Streptomyces} \) P450s have been reported. However, only a few studies were focused on their electron-transport proteins Fds and FdRs [29–31]. [2Fe-2S] Fd from \( \text{Pseudomonas putida} \) (CamB) plays a role in electron transfer from FdR from \( \text{P. putida} \) (CamA) to CYP107P3 of \( S. \text{griseus} \), and mediate the O-dealkylation of 7-etgoxycumarin [32]. CamB can also transfer electron to CamA and sustain the hydroxylation of daidzein by CYP107H1 from \( \text{Bacillus subtilis} \) [15]. Fd containing [3Fe-4S] cluster from \( S. \text{clavuligerus} \) was shown to be involved in clavulanic acid biosynthesis. \( \text{Mycobacterium tuberculosis} \) [3Fe-4S] Fd can pass electron to CYP51, a 14\(^{\alpha}\)-sterol demethylase [26]. Thirteen CYP107Z genes had been found till now in \( \text{Streptomyces} \), all of which exhibited regioselective oxidation activities to avermectin. Only Fd232 in \( S. \text{tubercidicus} \) I-1529 and Fd233 in \( S. \text{tubercidicus} \) R-922 were identified to be the electron transfer proteins of CYP107Zs for oxidation of avermectin. Both Fd233 and Fd232 are [3Fe-4S] Fds and have a high homogeneity (only one amino acid difference) and their flanking sequences are also of high homogeneity [3]. We speculated that Fd, which can transfer electrons to CYP107Z13 for oxidation of avermectin in ZB01, might be homologous to Fd232 and Fd233. Thus we designed primes F1/R1 according to the homologous flanking sequences of \( fd232 \) and \( fd233 \) and successfully cloned \( fd68 \) gene from ZB01 by PCR. We expressed \( fd68 \) in a prokaryotic expression system and purified a 7.1 kDa recombinant protein Fd68. The expression amount and the activity of purified Fd68 were relatively low (data not shown). This may partially due to the instability, small molecular weight and low expression level of Fd68 by \( \text{E. coli} \).

It was generally believed that the homologous double exchange mutants were more stable than single exchange mutants. We constructed \( fd68 \) homologous double exchange (data not shown) and single exchange gene disruption plasmids respectively by utilizing pKC1139, and only homologous single exchange mutants were obtained. Both the two \( fd68 \) gene disruption mutants \( ZB\Delta fd68-3 \) and \( ZB\Delta fd68-6 \) were genetically stable and lost the activity of oxidation avermectin, showing that Fd68 is a key electron transfer protein of oxidising avermectin by CYP107Z13 in ZB01.

Both of the oxidized FdR18 and FdR28 showed typical UV-visible absorption spectrum of FAD dependent enzyme [33], which are similar to that of FdR in \( \text{Pseudomonas putida} \) [34], ArR of \( \text{Nocophialobium aromaticivorans} \) [11], ApB in \( \text{Salmonella enterica} \) and FdRs in \( S. \text{coelicolor} \) A3(2) [35] and \( S. \text{Grisu} \) [9]. Both FdR18 and FdR28 are possibly NADH-dependent FdRs, which is similar to FdRs in \( S. \text{coelicolor} \) A3(2) and \( M. \text{tuberculosis} \) [26,36]. Both \( fdr18 \) and \( fdr28 \) knockout mutants in this study can oxidize avermectin to form 4\(^{-}\)-O-avermectin, although both the conversion efficiency

---

**Figure 4. Expression and characterization of FdR18 and FdR28.**

(A) Recombinant expression vectors pRSET-fdr18 and pRSET-fdr28. (B) SDS-PAGE analysis of recombinant proteins FdR18 and FdR28 expressed by \( \text{E. coli} \) BL21 (DE3). Mr: protein markers. (C) UV-visible spectra of purified FdR18 and FdR28. Spectra were recorded at ambient temperature in 50 mM Tris buffer (pH 7.5). (D) DCPIP reduction activities of purified FdR18 and FdR28, measured in the presence of 200 uM NADH (■) or NADPH (□).

doi:10.1371/journal.pone.0098916.g004
decreased about 46–60% comparing to that of wild ZB01 (data not shown). We had not got the fdr18-fdr28 double gene-disruption mutants. To determine whether FdR18 and FdR28 are electron transfer proteins in the catalytic reaction of oxidizing avermectin by CYP107Z13, we constructed two whole-cell biocatalytic systems co-expressing CYP107Z13, Fd 68 and FdR18/FdR28 in E. coli BL21 (DE3), using two compatible vectors pRSFDuet-1 and pRSF-1, and clarified that both of FdR18 and FdR28 could sustain the electron transfer activities to oxidise avermectin by CYP107Z13 [37,38].

FdR and Fd coding genes in Streptomyces may clustered with P450 genes, some of which are distributed freely in the genome. In the S. coelicolor A3(2) genome, fdr1, fdr4 with cyp105d5 and fdr2, fdr3 with cyp158a1 are located close together, but fdr5, fdr6, and the other four fds are located far from each other with other P450 genes [26]. Six fds and nine fds are present in S. avermitilis genome, only fdrB and fdrC with cyp105q1 are located close together [28]. fdr68, fdr18 and fdr28 were not clustered with cyp107z13. However, there is another unknown P450 gene at 49 bp upstream of fdr68 (data not shown), which hints that Fd68, FdR18 and FdR28 may not be the natural electron transport proteins for CYP107z13 in ZB01.

**Author Contributions**

Conceived and designed the experiments: ML XJ. Performed the experiments: YZ LZ. Analyzed the data: ML YZ. Contributed reagents/materials/analysis tools: YZ LZ XY. Wrote the paper: ML YZ.
References

1. Li M, Zeng F (2000) Research Progress of Streptomyces Cytochrome P450. Microbiol China 35: 1107–1112.
2. Choi KY, Jung EO, Yun H, Yang YH, Kazlauskas RJ, et al. (2013) Development of colorimetric HTS assay of cytochrome P450 for ortho-specific hydroxylations, and engineering of CYP102D1 with enhanced catalytic activity and regioslectivity. ChemBiochem 14: 1231–1238.
3. Bernhardt R (2006) Cytochromes P450 as versatile biocatalysts. J Biotechnol 124(1): 128–145.
4. Mohran I, Hill DS, Zirkle R, Hammer PE, Gross F, et al. (2005) Biocatalytic conversion of avermectin to 4'-oxo-avermectin: heterologous expression of the euk cytochrome P450 monoxygenase. Appl Environ Microbiol 71: 6977–6985.
5. Mohran I, Jungmann V, Stege J, Trefzer A, Pachlathko JP (2006) Biocatalytic conversion of avermectin to 4'-oxo-avermectin: discovery, characterization, heterologous expression and specificity improvement of the cytochrome P450 enzyme. Biochem Soc Trans 34: 1236–1240.
6. van Beilen JB, Funhoff EG, van Loon A, Just A, Kaysser L, et al. (2006) Cloning and prokaryotic expression of a CYP153 alkane hydroxylase gene in a Gram-positive Dietzia sp. DQ12-45-1b and characterization of the specific NADH: ferredoxin oxidoreductase of this cytochrome P450 system. Microbiol 151: 2985–2003.
7. Yong N, Jie-Liang L, Hui F, Yue-Qin T, Xiao-Lei W (2014) Characterization of a CYP153 alkane hydroxylase gene in a Gram-positive Dietzia sp. DQ12-45-1b and its “team role” with alkW1 in alkane degradation. Appl Microbiol Microbiol 72: 59–65.
8. Bell SG, Tan AB, Johnson EO, Wong LL. (2010b) Selective oxidative demethylation of veratic acid to vanillic acid by CYP195A1 from Rhodotorula glutinis (A) HaA2. Mol Biocyt J 6(1): 206–214.
9. Ramachandra M, Seetharam R, Emptage MH, Sariaslani FS (1991) Purification and characterization of a soybean flour-inducible ferredoxin reductase of Strepomyces griseus. J Bacteriol 173: 7106–7112.
10. Kieser M, Hannemann F, Butter M, Zapp J, Bernhardt R (2012) CYP105A1-mediated 3-hydroxylation of glimepiride and glibenclamide using a recombinant Bacillus megaterium whole-cell catalyst. J Biotechnol 157: 405–412.
11. Bell SG, Dale A, Rees NH, Wong LL. (2010) A cytochrome P450 class I electron transfer system from Nonobullebium amaraeacutens. Appl Microbiol Microbiol 86: 163–173.
12. Chen YJ, Shimada T, Waterman MR, Guengerich FP (2006) Understanding electron transport systems of Streptomyces cytochromes P450. Biochim Biophys Acta 1766: 180–194.
13. Green AJ, Munro AW, Chresman MR, Reid GA, Wachenfeldt von C, et al. (2003) Expression, purification and characterization of a Bacillus subtilis ferredoxin: a potential electron transfer donor to cytochrome P450. Biochim Biophys Acta 1577: 92–98.
14. Shrestha P, Oh TJ, Sohn JK (2006) Cloning and expression of a CYP153A11 oxidase from a monocyclic hydroxylating whole-cell biocatalyst. J Mol Biol 357: 241–253.
15. Choi KY, Jung EO, Yun H, Yang YH, Kazlauskas RJ, et al. (2013) Whole-cell-based CYP153A6-catalyzed (S)-limonene hydroxylation efficiency and characterization of the specific NADH: ferredoxin oxidoreductase of this cytochrome P450 system. Microbiol 151: 2985–2003.
16. Liu WD, Jiang XL, Ji Y, Niu J, Li M (2011) Cloning and prokaryotic expression of cyp107z gene from Streptomyces avermitilis ZB01. Acta Microbiol Sin 51: 40–46.
17. Jiang XL, Liu WD, Ji Y, Niu J, Li M (2012) Expression of CYP107Z13 in Streptomyces lividans TK4 catalyzes the oxidation of avermectin to 4'-oxo-avermectin. Appl Microbiol Biotechnol 93: 1957–1967.
18. Kieser T, Bibh MJ, Butter MJ, Chater KF, Hopwood DA (2000) Practical Streptomyces genetics. The John Innes Foundation, Norwich.
19. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
20. Biermann M, Logan R, O’Brien K, Seno ET, Rao RN, et al. (1992) Plasmid cloning vectors for the conjugal transfer of DNA from Escherichia coli to Streptomyces spp. Gene 116: 43–49.
21. Hopwood DA, Bibh MJ, Chater KF, Kieser T, Bruten CJ, et al. (1985) Genetic manipulation of Streptomyces: A laboratory manual. The John Innes Foundation, Norwich.
22. Kirsty JM, Nigel SS, Andrew WM (2003) Kinetic, spectroscopic and thermodynamic characterization of the Mycobacterium tuberculosis adrenodoxin reductase homologue FpaA. Biochem J 372 (Pt 2): 317–327.
23. Hanahan D (1983) Studies on transformation of Escherichia coli with plasmids. J Mol Biol 166: 557–580.
24. Sevrioukova IF, Poulos TL (2011) Structural biology of redox partner binding proteins. J Bacteriol 193: 887–895.
25. Sielaff B, Andreesen JR (2005) Analysis of the nearly identical morpholine reductases in Mycobacterium tuberculosis. J Mol Biol 4: 557–580.
26. Lamb DC, Bieda H, Nelson DR, Ishikawa J, Skag T, et al. (2005) Cytochrome P450 complement (CYPome) of the avermectin-producer Streptomyces avermitilis and comparison to that of Streptomyces coelicolor A3(2). Biochim Biophys Res Commun 307: 610–619.
27. Hopwood DA, Bibh MJ, Chater KF, Kieser T, Bruten CJ, et al. (1985) Genetic manipulation of Streptomyces: A laboratory manual. The John Innes Foundation, Norwich.
28. Parajuli N, Basnet DB, Lee HC, Sohn JK, Liu K (2004) Genome analysis of Streptomyces peucetius ATCC 27592 for the identification and comparison of cytochrome P450 complement with other Streptomyces. Arch Biochem Biophys 425: 231–241.
29. Pramod S, Tao-Jin O, Jae KS (2008) Desigining a whole-cell biotransformation system in Escherichia coli using cytochrome P450 from Streptomyces piceacin. Biochim Biophys Acta Lett 30: 1100–1106.
30. Joo YC, Jeong KW, Yeom SJ, Kim YS, Kim Y, et al. (2012) Biochemical characterization and FAD- binding analysis of oleate hydrolase from Macrococcus communis. Biocymic 94: 907–915.
31. Peterson JA, Lorence MC, Anareh B (1999) Putidaredoxin reductase and putidaredoxin: cloning, sequence determination, and heterologous expression of the proteins. J Biol Chem 265: 6066–6073.
32. Boyd JM, Endrissi JA, Hamilton TL, Christopherson MR, Mulder DW, et al. (2011) FAD binding by ApeF protein from Salmonella enterica: a new class of FAD-binding proteins. J Bacteriol 193: 897–893.
33. Qiao F, Zhang JM, Bai YL, Yang XY, Li CR, et al. (2012) Analysis of the role of FdhA and FdrA in CYP125A1’s electron transfer chain, two ferredoxin reductases in Mycobacterium tuberculosis. Chin Med Biotechnol 7: 178–186.
34. Duetz WA, van Beilen JB, Wirthb B (2001) Using proteins in their natural environment: potential and limitations of microbial whole-cell hydroxylations in applied biocatalysis. Curr Opin Biotechnol 4: 419–425.
35. Huang MH, Tian YQ, Lu Q (2010) Protease electron transport pathway and whole-cell transformation system in Streptomyces P450. J Microbiol 1: 75–79.