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Research Article

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Cloning and sequence analysis of a novel NADPH-cytochrome P450 reductase gene from filamentous fungus *Curvularia lunata*

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ABSTRACT

Microbial transformation is an essential component for the commercial production of numerous valuable steroid drugs such as hydrocortisone, prednisolone. Steroid hydroxylation at C11 and C15 positions by filamentous fungi is routinely employed in the steroid industry due to both the necessity of regio- and stereoselective oxyfunctionalization of unactivated C-H bonds of valuable steroid compounds for their desired pharmaceutical activities and also the difficulty of accomplishing such tasks by conventional chemical means. To characterize the steroid hydroxylation system in filamentous fungus Curvularia lunata, which is capable of incorporating a single oxygen atom at C11-β of cortexolone and other derivatives, a NADPH-cytochrome P450 reductase gene was cloned from the fungus based on transcriptome analysis and PCR amplification. The cloned cytochrome P450 reductase gene is predicted to encode a polypeptide of 695 amino acids and exhibits strong similarity to NADPH-cytochrome P450 reductases of several Aspergillus species, with the highest similarity to Aspergillus nidulans observed. The fungal NADPH-cytochrome P450 reductase is an indispensible partner of the microsomal cytochrome P450 hydroxylase by acting as an electron donor. Given the great value of effective 11-β hydroxylation of various steroid compounds for commercial production of valuable steroidal intermediates and drugs, it is anticipated that the cloning and further characterization of the NADPH-cytochrome P450 reductase from C. lunata should facilitate the engineering of industrial microbial strains for efficient drug relevant steroid hydroxylation.

Key words: *Curvularia lunata*, P450 reductase, steroid drug, 11-β hydroxylation, microbial transformation

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INTRODUCTION

Cytochrome P450s constitute a superfamily with a wide broad range of catalytic activities such as hydroxylation, epoxidation, dealkylation, heteroatom oxygenation, aromatic hydroxylation, reduction, and dehalogenation [1]. The biocatalytic versatility of P450s has attracted broad attention for their potential biotechnological applications in various industrial sectors for production of fine chemicals, biofuel, bulk chemicals, pharmaceuticals etc or as a means for bioremediation [2,3]. In the pharmaceutical industry, a number of filamentous fungal cytochrome P450s systems have been widely used as catalysts for regio- and stereoselective steroid hydroxylation and serve as a key component for the commercial production of valuable steroidal drugs since the late 1950s [4,5]. Important fungal catalysts include *Rhizopus nigricans*, *Aspergillus ochraceus* [6] and *Aspergillus niger* for steroid C11-α hydroxylation, *Absidia coerulea* and *Curvularia lunata* for C11 β - hydroxylation [7,8]. The fungal cytochrome P450s hydroxylating system is believed to consist of two components, a microsomal membrane bound cytochrome P450 hydroxylase (CYP) and its electron donor of a micromal NADPH-cytochrome P450 reductase (CPR) containing FAD and FMN [9,10,11]. However, the molecular basis underlying its regio- and stereoselective catalytic activities is poorly understood. Although the apparent importance of fungal steroid hydroxylation systems, few genes encoding the fungal cytochrome P450 hydroxylase and the NADPH-cytochrome P450 reducatse have been cloned and characterized, thus hampering attempts to improve the steroid hydroxylation catalysts by targeted genetic manipulation.

To identify the NADPH-cytochrome P450 in *C. lunata*, we analyzed the RNA sequencing data of the transcripts extracted from fungal mycelia under steroid induction. In this work, we present evidence that the cloned gene encodes a novel NADPH-cytochrome P450 reductase.

EXPERIMENTAL SECTION

Strains, media, and growth conditions

Filamentous fungus *C. lunata* AS3.3589, obtained from the microbial strain collection of the applied microbiology lab of Tianjin University of Science and Technology, was routinely maintained on potato dextrose agar (PDA) medium. To obtain mycelia for total RNA extraction, strain AS3.3589 was cultured overnight at 28℃ in liquid medium containing: 2% glucose, 0.5% yeast extracts, 0.5% peptone, 1% soybean power, pH 6.5, followed by adding 0.01% cortexolone for induction and continuing cultivation for 6 h.

RNA isolation, cDNA synthesis, and PCR amplification

Total RNAs were isolated using Trizol reagents (Promega, USA) from the *C. lunata* culture treated with 0.01% (W/V) cortexolone for 6 hr, and reverse transcription of the first cDNA strand was performed using 3 ug of total RNA with PrimeScript Reverse Transcriptase (TaKaRa, Dalian, PR China) in a 20 ul reaction volume according to the manufacturer's instructions. A 1-ul aliquot of cDNA was used as the template for PCR amplification of cDNA of the NADPH-cytochrome P450 reductase gene on 7900 system (Applied Biosystems Inc, USA) with the following primer set:

ACT-F: 5'-CTTTTCAACAAGCACCATCTGC-3' (forward),
ACT-R: 5'-TCATTCTCGGTTTGTAGGTCGTCG-3' (reverse).

The amplifications were conducted in a total volume of 20 ul, containing 1ul of SYBR green (CWBIO, Beijing, PR China), 300 nmol/liter of both primers, and 1 ul of cDNA. The amplification was conducted as follows: 10 min at 95°C, followed by 30 cycles consisting of 15 s at 95°C, 30 s at 60°C and 2 min at 72°C. The transcript level of actin gene was used as an internal control using the primer pair: CPR-F-Bgl Ⅱ:

5'-AGATCTATGTCTCAACTCGACACTC-3'(Bgl Ⅱ site underlined);
CPR-R-Hind Ⅲ：5'-AAGCTTTCATGACCAGACGTCCTC-3' (Hind Ⅲ site underlined).

Sequence analysis and phylogenetic tree construction

Sequence analysis for homology comparison was performed with DNAMAN V6 (Lynnon Biosoft). Conserved domain search was conducted with CD search at the NCBI site. Phylogenetic analysis was conducted using the MEGA6 software downloaded from the website: www.megasoftware.net.

RESULTS AND DISCUSSION

Cloning of a cytochrome P450 reductase from filamentous fungus C. lunata

To identify genes involved in the steroid cortexolone hydroxylating activities, transcripts expressed under cortexolone induction were RNA sequencing. Transcriptome data mining revealed a transcript encoding a cytochrome P450 reductase of 695 amino acids. A set of primers was designed to amplify its predicted whole ORF from cDNA synthesized from total RNA extracted from the fungal culture treated with 0.01% (W/V) cortexolone for 6 h. As shown in figure 1, a cDNA fragment of about 2.1 kb was amplified, consistent with the predicted size of the P450 reductase gene.

Sequence analysis of the C. lunata cytochrome P450 reductase gene

PCR products of the ORF of the cytochrome P450 reductase gene was cloned into T-vector, followed by sequencing. Sequence analysis of the predicted amino acid sequence of the NADPH-cytochrome P450 reductase revealed that it contains a conserved flavodoxin domain covering the region from 68-216 residues and FAD binding domain spanning residues 273-491, characteristic structure of NADPH-cytochrome P450 reductase. As shown in figure 2, sequence comparison showed that the *C. lunata* cytochrome P450 reductase displays very high identity with a NADPH-cytochrome P450 reductase from *Aspergillus nidulans* (XP 658199), bearing 92% identity over the whole protein of 695 residues. In addition, NCBI blast search also revealed that this novel P450 reductase exhibits strong homology with NADPH-cytochrome P450 reductases from several other Aspergillus species, including *A. niger*.

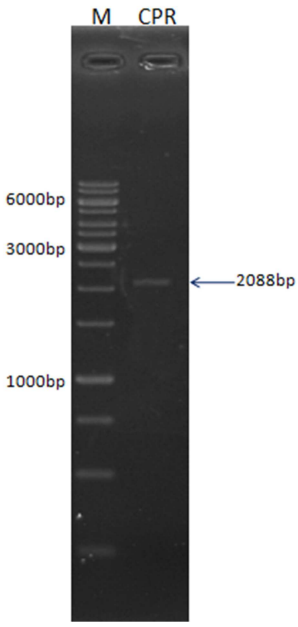

Fig. 1. Electrophoresis of PCR products of the *C. lunata* P450 reductase gene ORF on 0.8% agarose gel

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upper line:CPR Curvularia lunata AS3.3589
lower line:CPR Aspergillus nidulans FGSC A4

61  ETGKNCVIFYGSQTGTAEDYASRLAKEGSQRFGLKTMVADLEEYDYENLDKFPDDKVAFF
    |||||||||||||||||||||||||||||||||||||||:||||||||||.|||:|||||||
61  ETGKNCVIFYGSQTGTAEDYASRLAKEGSQRFGLKTMVADIEEYDYENLDQFPEDKVAFF
                          Flavodoxin domain(68-216aa)
121 VMATYGEGEPTDNAVEFYQFLTSEDVSFEGGGSADDQPLSSLKYVIFGLGNNTYEHYNAM
    |:|||||||||||||||||||:|:::||||||||||:|.||||||||  ||||||||||||||
121 VLATYGEGEPTDNAVEFYQFITGDDVSFEGGGSAEDKPLSSLKYVAFGLGNNTYEHYNAM

181 VRQVDAAFTKLGAQRIGSAGEGDDGAGTMEEDFLAWKEPVWAALSESMGLQERDASYEPV
    |||||||:|||||||||||||||||||||||||||||||:|||||||.|.||||:|||||||
181 VRQVDAALTKLGAQRIGSAGEGDDGAGTMEEDFLAWKEPMWAALSEAMNLQEREASYEPV

241 FNVTEDDSLNPEDASVYLGEPTKGHLDGEAKGPFSAHNPFIAPIVESRELFTVKDRNCLH
    | ||||:||.|||.||||||||||||:|:::.||:||||||:||||||||||||||||||||
241 FCVTEDESLTPEDNSVYLGEPTKGHLEGQPNGPYSAHNPYIAPIVESRELFTVKDRNCLH

301 MEISIAGSGLSYQTGDHIAVWPTNAGAEVDRFLSVFGLEEKRNSVISIKGIDVTAKVPIP
    |||||||..|.|||||||:|||||||||||.|||||||||:|||.||||||||||||||||
301 MEISIAGTNLTYQTGDHIAIWPTNAGAEVDRFLNVFGLEEKRHSVINIKGIDVTAKVPIP
                          FAD binding domain(273-491aa)
361 TPTTYDVAVRYYMEVCAPVSRQFVATLAAFAPDEESKAEIVRLGSDKDYFHEKITNQCFN
    ||||||.|||||||||||||||||.||||||||||.|.|||||||||||||||||||||||
361 TPTTYDAAVRYYMEVCAPVSRQFVSTLAAFAPDEETKTEIVRLGSDKDYFHEKITNQCFN

421 IAQALQTITSKSFTNVPFSLLVEGLNKIQPRYYSISSSSLVQKDKISITAVVESTRLPGA
    ||||||.||||.|.|||||||:|||||||||||||||||||||||||||||||||||||||
421 IAQALQSITSKPFSNVPFSLLIEGLNKIQPRYYSISSSSLVQKDKISITAVVESTRLPGA

481 THVVKGVTTNYLLALKQKQNGDPSPDPHGQTYDITGPRNKYDGIHVPVHVRHSNFKLPSD
    ||:|||||||||||||||||||||||||||||||.|.|||||||||||||||||||||||||
481 THIVKGVTTNYLLALKQKQNGDPSPDPHGQTYAINGPRNKYDGIHVPVHVRHSNFKLPSD
```

Fig. 2. Highly conserved flavodoxin domain and FAD binding domain between the NADPH-cytochrome P450 reductases of *C. lunata* and *A. nidulans*

Phylogenetic analysis of the C. lunata NADPH-cytochrome P450 gene

To better understand the relationship between the cloned *C. lunata* NADPH-cytochrome P450 reductase and its counterparts in other filamentous fungi, its phylogenetic relationship with 9 predicted P450 reductases with various similarities were analyzed with MEGA6 software [12]. As shown in figure 3, the *C. lunata* P450 reductase is closely related with P450 reductases from *A. nidulans* and *A. niger*.

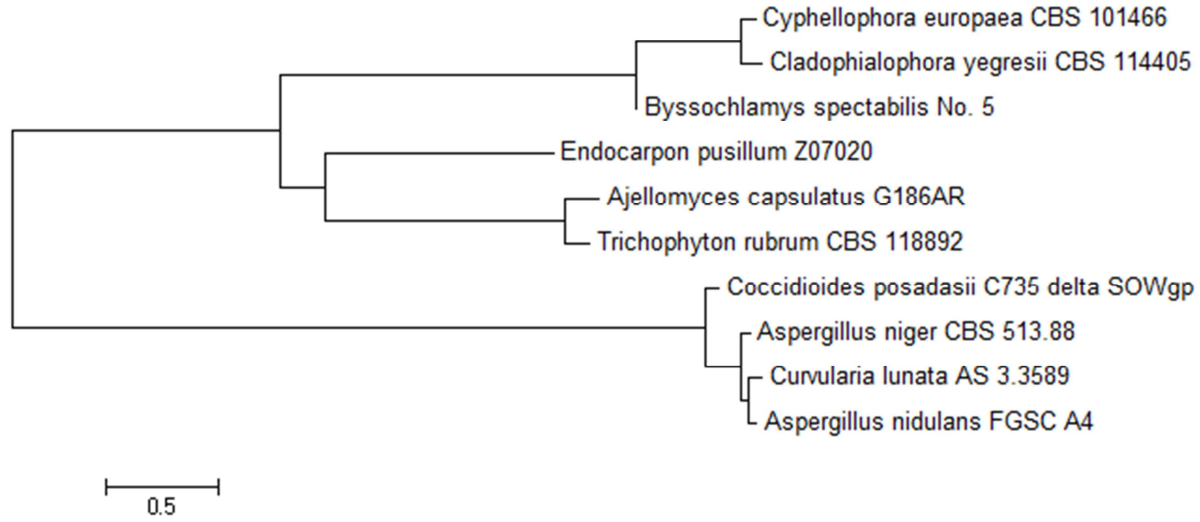

Fig. 3. The phylogenetic relationship of NADPH-cytochrome P450 reductases between *C. lunata* and 9 other filamentous fungal species

CONCLUSION

The function of fungal microsomal cytochrome P450 requires that the corresponding NADPH-cytochrome P450 reductase functions as an electron donor. Filamentous fungi are a rich resource of cytochrome P450 systems capable of hydroxylating steroids at positions relevant to steroid pharmaceutical activities, such as C11-α and C11-β hydroxylation. Filamentous fungus *C. lunata* AS3.3589 possesses steroid C11-β hydroxylation activities, however, little is known about its cytochrome P450 system involved in hydroxylation reactions. The cloning and further characterization of the *C. lunata* P450 reductase will provide insights into the mechanistic aspects of its P450 steroid hydroxylating system. Moreover, the availability of this P450 reductase will facilitate the engineering of *C. lunata* P450 steroid C11-β hydroxylation capability in heterologous systems to develop more efficient processes for steroid production.

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