Expression and characterization of two new alkane-inducible cytochrome P450s from

R. Del Carratore, P. G. Gervasi, M. P. Contini, P. Beffy, B. E. Maserti, G. Giovannetti, A. Brondolo, V. Longo

Section: Microbial and Enzyme Technology

Expression and characterization of two new alkane-inducible cytochrome P450s from *Trichoderma harzianum*

R Del Carratore[1], PG Gervasi[1], MP Contini[1], P Beffy[1], BE Maserti[3], G Giovannetti[4], A Brondolo[4], V Longo[2]

[1]Institute of Clinical Physiology, National Research Council (CNR), via Moruzzi 1, Pisa, Italy,

[2] Institute of Agricultural Biology and Biotechnology, CNR, via Moruzzi 1, Pisa, Italy.

[3] Institute of Biophysics, CNR, via Moruzzi 1, Pisa, Italy

[4] Experimental Culture Centre Valle D'Aosta, Fraz. Olleyes, 9 Quart (AO), Italy

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Abstract

n-Dodecane and fatty acids were good inducers of cytochrome P450 (CYP) and the ω-hydroxylase of lauric acid, which is a marker for ω-hydroxylation of n-alkanes, in *Trichoderma harzianum.* A cDNA, containing an ORF of 1520 bp, encoding a CYP52 of 520 amino acids, was isolated by RACE. Another n-alkane-inducible CYP was identified by LLC-MS/MS analysis of a microsomal protein band induced by n-dodecane in a library of *T. harzianum*. This suggests that *T. harzianum* has a CYP-dependent conversion of alkanes to fatty acids allowing their incorporation into lipids.

Introduction

Microbial monooxygenase hydroxylase enzymes involved in the metabolism of endogenous and xenobiotic compounds have potential application in many areas such as bioremediation of petroleum, hydrocarbon contamination (Lynch et al., 2005) and organic synthesis (Beilen et al., 2005). However cytochrome P450 (CYP)-dependent monooxygenases in filamentous fungi have not yet received adequate attention (Vatsyayan et al., 2008). *Trichoderma* species, which are present in nearly all soils (Yedidia et al., 1999), are involved in plant defense, and some strains are able to readily colonize plant roots.

Various members of the CYP52 family are alkane-inducible with major isoforms being involved in the degradation of alkanes via terminal hydroxylation. The derived alcohols are converted to fatty acids which enter into the ß-oxidation process. Indeed, several CYP isoforms of this family have been reported in the alkane-assimilating yeasts such as *Yarrowia lipolytica* and *Candida maltosa* (Zimmer et al., 1998; Fickers et al., 2005). However, studies on the monooxygenase system of *Trichoderma* species have not yet been reported. Here we present the cloning and the sequence of a new member of the CYP52 family in *T. harzianum* strain and its inducibility by alkanes. In addition, the presence and inducibility of another CYP form is described.

Materials and methods

Chemicals

Gene Racer kit and pyrenyl-diazomethane were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). All the other chemicals were supplied by Sigma.

Fungal strain classification

Trichoderma harzianum was isolated from polycyclic aromatic hydrocarbons-contaminated soil (Free PCB project Life 03/ENV/IT/000321) and was identified by a specific "barcode" developed on the basis of 285 voucher sequences corresponding to 5.8 S rRNA gene using the primers ITS1 and ITS4 (Druzhinina et al., 2005). The strain was identified with an high score as *Hypocrea lixii/T. harzianum* by sequence comparison using a program for the oligonucleotide barcode based species identification TrichOKEY v 1.0, (http://isth.info/index.php).

Microsomal preparation

Frozen mycelia were disrupted by grinding in liquid N_2 and subsequently suspended in 0.1 M HEPES buffer containing 20% (v/v) glycerol, 1 mM EDTA, 1mM dithiothreitol, 25 mM PMSF, 1 μM FAD, and 1 μM FMN, pH 7.2 (Buffer A). Microsomes were prepared and suspended in Buffer A at 6-8 mg protein/ml and solubilized in CHAPS according to Messina et al., (2010).

CYP content and enzymatic assays

The CYP content was determined in solubilized microsomes by the method of Omura and Sato (1964). The microsomal lauric acid hydroxylase activity marker for CYP 52 isoforms, was assayed by HPLC, after metabolite derivatization with pyrenyl-diazomethane (see Zanelli et al., 1996).

RNA extraction and RT-PCR

Total RNA extraction from 10 mg frozen mycelium was performed modifying the method suggested for the TaqMan Gene Expression Cells-to-CT TM Kit (Applied Biosystems).

CYP52 cloning and sequencing

PCR primers used in this study are listed in the Supplementary Table 1. To isolate CYP52 genes we designed degenerate PCR primers based on alignment of the conserved sequences, such as the I-helix and heme-binding regions (Craft et al 2003), of four alkane hydroxylase CYP isoforms (namely CYP52G1, 52G2, 52H1 and 52H2) of *Aspergillus fumigatus* and *A. nidulans*. A PCR fragment of about 650 bp was detected by degenerated primers dF and dR in the cDNA from *T. harzianum* treated with 0.5 % n-dodecane. On the basis of this fragment sequence, we designed two specific primers F1 and R1 which amplify a PCR fragment of about 500 bp. Genome-wide analysis of the isolated cDNA shotgun database (http://blast.ncbi.nlm.nih.gov.) revealed that our cDNA fragment had an homology of about 80 % with the fragments FG 375146.1 and NGE 789708.1 of *Trichoderma virens*. The full-length cDNA was obtained by RACE procedure. For isolation of the missing 5'-end, the reverse primers: 5'RP (racer primer) and 5'NP (nested primer) were used; whereas for isolation of the missing 3'-end, the forward primers 3'RP (racer primer) and 3'NP (nested primer) were used. The Gene Racer products were sub-cloned in pGEM-T easy vector and sequenced by BMR Genomic Sequencing Core Service (Padova, Italy). The transcription level of β-actin gene, as a control gene, was amplified with the primers: Fa and Ra.

SDS-PAGE and LC-ESI MS/Ms analysis

The band of 50-58 kDa in the SDS-PAGE gel was cut and processed using a MultiProbe II liquid handling automated (PerkinElmer). LC-MS/MS protein analysis was performed by Proteomique Platform (INRA, Montpellier). Raw data were processed using the Mascot software (Matrix Sciences) in the Lamda Zap Library of *T. harzianum.*

Results and discussion

CYP induction and activity

Mycelium (100 mg) was inoculated in 400 ml (in a 1 l flask) of nutrient-limited potato/dextrose broth (PDB) medium containing 0.1% glucose at 25° C for 60 h. Maximum yield was about 6 g dry wt/flask (Fig. 1). Addition of alkanes as a carbon source after 40 h from the mycelial inoculum increased the final yield (e.g. n-dodecane addition gave about 7.5 g as shown in Fig.1). Six h after alkane addition, mycelia were collected and processed for CYP detection and activity assays.

Insert Fig.1 and Fig.2

P450 was not detected in cells grown in nutrient-limited PDB medium. Induction of CYP by various n-alkanes (n-decane, n-dodecane, n-tetradecane, n-octadecane), fatty acids or a mixture of n-dodecane and fatty acids is shown in Fig. 2. To verify if CYP induction by alkanes was accompanied by the induction of specific isoforms (i.e. CYP52) able to perform ω-oxidation of hydrocarbons, the ω-hydroxylation of lauric acid by microsomes, obtained from n-alkane- or fatty acids-treated *T. harzianum* cultures, was determined. Treatment with the mix containing both 0.2 % fatty acids and 0.5 % n-dodecane was the most effective (Fig. 3). These results suggest that one or more induced CYP52 genes in *T. harzianum* may convert alkanes to fatty acids and to incorporate them into cellular lipids as in other aerobic, alkane-degrading microorganisms.

Insert Fig.3

CYP52 gene cloning, sequence analysis and expression

Using the PCR approach followed by 5'- and 3'-rapid amplification of cDNA (RACE), a full-length CYP52 cDNA of 1770 bp was isolated from a 0.5 % n-dodecane treated culture of *T. harzianum*. This cDNA contained an ORF of 1520 bp flanked by the initiation (ATG) and termination (TAG) codons and by the 5'- (79 bp) and 3'- (128 bp) non-coding regions (Supplementary Figure1). The cDNA encoded a protein of 520 amino acids which had a calculated Mr of 58975 Da and a pI=7.66. The amino acid sequence contained an heme-binding peptide (FNGGPRICIGQQFA) positioned between amino acid residues 459 and 472 (see box in Fig. 4), and the invariant cysteine, responsible for the fifth ligand to the heme iron at position

466. The 14 aa of heme binding region is found in many other fungal CYP 52. When we compared the amino acid sequence of the cloned CYP of *T. harzianum* with the CYP enzymes of the three existing genome sequences of *Trichoderma* species, an homology of about 90, 80 and 75 %, with the sequences of estExt_Genewise1.C_530012 in the *T. virens*, of fgenesh5_pg.C_scaffold_1000780 in the *T. reesei*, and of estExt_Genewise1.C_90314 in the *T. atroviride*, respectively, was found. In addition, at least another ten CYPs were found in each *Trichoderma* species, showing a lower homology (between 30 and 45%) with our CYP52. These results indicate that the cloned CYP belongs to the 52 family and, after submission to Dr. DR Nelson (Cytochrome P450 Homepage, Nelson 2009) it has been designed at - as CYP52X3. By RT-PCR we showed that this gene was transcriptionally induced in the *T. harzianum* cultures treated with both 0.5% n-dedecane and 0.25% fatty acids (Fig. 5), in keeping with the enhanced activity of ω-lauric acid hydroxylase previously described.

Insert Fig. 5 and Fig. 6

Microsomal CYP analysis

In order to detect if other CYPs are induced by n-dodecane, SDS-PAGE of microsomes obtained from control and 0.5 % n-dodecane-treated *T. harzianum* cultures was performed. The protein band, 50 to 58 kDa (Fig. 6), was excised and analyzed by LC-ESI MS/MS. Several peptides were identified in this band and among them, three resolved peptides (namely: MSPSVGGILPR, AIGVADPSEGR, and KGPIVEFK) are indicated in bold in Fig. 7, matched, with a score of 108 to the unknown protein derived from the nucleotide sequence of 688 bp (AJ900936) of the mycelium Lambda Zap Library L10 *Hypocrea lixii* cDNA clone L10T34P112R09986 (http://blast.ncbi.nlm.nih.gov/Blast.cgi). This sequence is the COOH-terminal of a CYP containing the CYP signature (GPRGCIGKG) positioned between 137 and 146 amino acid residues (boxed in Fig. 7).

Insert Fig. 7 and Fig.8

This form had a sequence identity of 63 % in comparison with the CYP52A6 (EEY 17329.1) of *Verticillum albo-atrum* (Ma et al. 2008). The result suggests that this is an unknown CYP, temporarily designed as CYP548A10 with 70% amino acid identity to CYP548A4 of *Nectria haematococca* (Dr. DR Nelson, personal communication), which represented the major isoform in microsomes from n-dodecane-treated fungi, may be involved in the oxidation of n-alkanes. Indeed, the transcript of CYP548A10, when analysed by RT-PCR utilizing the pair of primers FN and RN (underlined in Supplementary Figure 2.), was strongly induced by 0.5 % n-dodecane (Fig. 8) suggesting that this gene might represent another important alkane-metabolizing CYP in *T. harzianum*.

Conclusions

We have demonstrated that *T. harzianum* contains at least two ER membrane-bound CYP enzymes inducible by n-alkanes, which can perform ω-oxidation of fatty acids and n-alkanes. This property may be useful in environmental applications.

Acknowledgments

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Figure captions

Fig. 1. *T. harzianum* growth curves with 0.1% glucose (■) or 0.1% glucose plus the 0.5 % n-dodecane treatment **(□). T**he treatment was done after 40 h of growth as indicated by the arrow**.** Mycelium was harvested at various times and dried to measure the weight.

Fig. 2 CYP content in microsomes obtained from *T. harzianum* cultured in nutrient-limited potato/dextrose broth in the presence of 0.03% (□), 0.25% (▩), 0.5% (▨) or 1% (▨) of each indicated compound. The Mix is composed of an equal amount of n-dodecane and fatty acids. The induction of CYP is depended on the n-alkane chain length showing the following rank order: n-dodecane>n-tetradecane>n-octadecane>n-decane. Each value, expressed as pmol/mg protein, represents the mean ± SD (bar) of three independent experiments.

Fig. 3 ω-Lauric acid hydroxylase activity in microsomes obtained from *T. harzianum* grown in the presence of three different n-dedecane-fatty acids mixtures: Mix 1 (0.1 % n-dodecane plus 0.1 % fatty acids), Mix 2 (0.5 % n-dodecane plus 0.1 % fatty acids) and Mix 3 (0.5 % n-dodecane plus 0.2 % fatty acids). The derivatized ω-hydroxy lauric acid was resolved by HPLC with a C18 RP Supelco column (250 x 4.6) using a linear gradient water: methanol (from 25:75 to 12:88 v/v) over 16 min at a flow 1.2 ml/min. Each value represents the mean ± SD (bar) of three independent experiments.

Fig. 4 Deduced amino acid sequence of *T. harzianum* CYP52. The often conserved aa residues in the other CYP52 (for example CYP52A12 and A13 of *Debaryomyces hansenii*) are boxed and the heme region is in bold and boxed. The helix I region is underlined.

Fig. 5 RT-PCR analysis of CYP52 and β-actin mRNA expression in control (C), 0.5 % n-dodecane (n-D) or 0.25% fatty acids-treated *T. hartzianum* cultures.

Fig. 6 SDS-PAGE of microsomal proteins (50 μg) of control (C) and 0.5 % n-dodecane-(n-D)-treated *T. hartzianum* cultures. SDS-PAGE was conducted using the method of Laemmli with 7.5% (v/v) acrylamide and stained with Coomassie Blue R-250. Protein markers in kDa (M) are shown.

Fig. 7 Deduced amino acid sequences of a CYP protein fragment of *T. harzianum.* The heme region is in bold faced and boxed. The peptides identified in the protein band are in bold faced.

Fig. 8 RT-PCR analysis with 23, 26 and 29 cycles of an unknown CYP and β-actin mRNA expression in control (C) and 0.5 % n-dodecane-(n-D)-treated *T. harzianum*.

Fig. 1

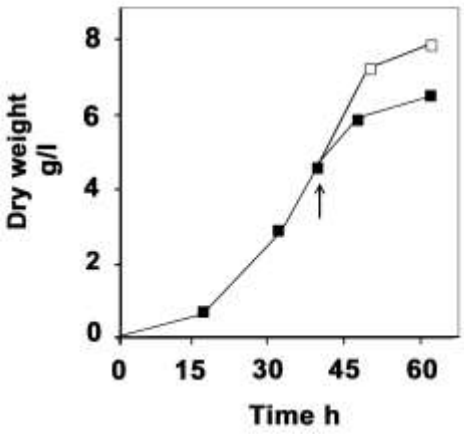
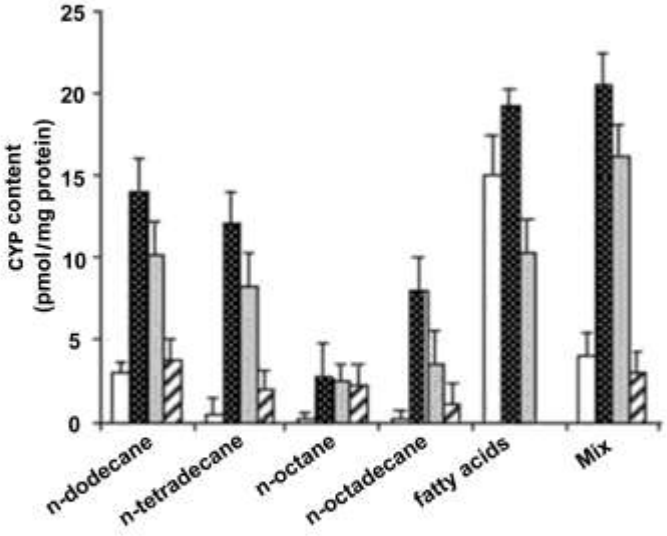


Fig. 2



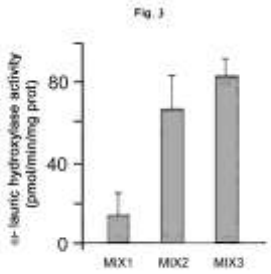


Fig. 4

MQLSLAAGIGAAALTFVYVLNLILESFRYRARSRELGCKPPVWMSGYDPTGI
ADIVLGVDASRKKRFPIHAADNFEREEKKHGRTVGTLRVKTPLFRDTLVTMD
PQNIQTILALKFKDFGLGVNRTENFEPLLGHGIFATNGKQWEHSRALLRPQF
IRGQVSDLNLEEEHVKSLMTVLNRQVGPDGWTDLVDLLPLFFRLTLDSATEF
LFGESVNSQLDQVHPTSEKDGNFAFAFDKAQYNLSIGARLGPNYWVVHTPEF
KRMVKRVHAYVDYFVQKALVQGEKMPVNEDKYVFLNALAKETRDPEELRSQL
LNILLAGRDTTASTLGWFFYTMADPAYEPVFRRLRAIILEEFGTYSNPKEIT
FEKMKGCQYLQWCLNECLRLYPAVPMNVRTAQVDTTLPIGGGPDGQSPIFVP
KGQDVGYSVHLMHRRKDIWGSDAEIFKPERWETRRPGWDYLP**FNGGPRICIG**
QQFALTEVGYVVIRLMQRIDNIDGSQVGPIKHGLTLTNCPGEGVNVKLHFAE

Fig. 5

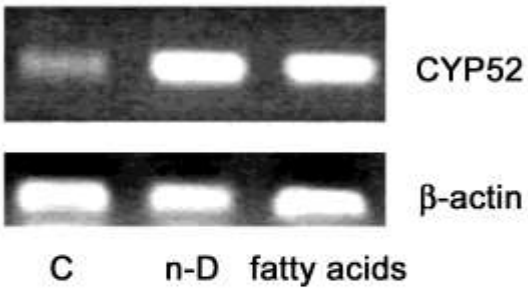


Fig. 6

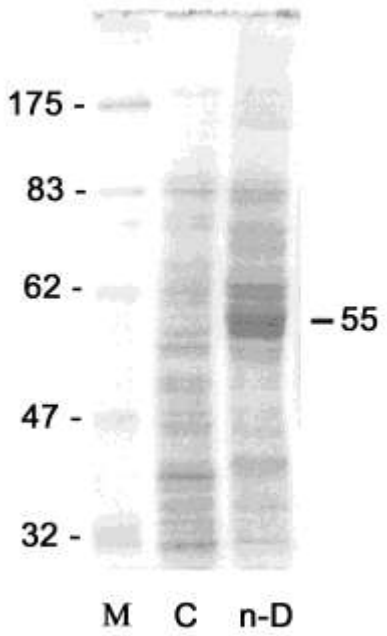


Fig. 7

STAVAAALELVDPPGCRNSARGSDVEEIVQGPTLASCTYLRACIDEAMR**MSPSVGG**
ILPREVLPGGITIDGHFVPAGIVVGTPHYNIHHNEEYYPQPFTYAPERWIKDAINP
ATKRESTVEDVAQAQAAFCPFSIG**PRGCIGKGLA**YVEMTLTLARAIYLYDMRR**AIG**
VADPSEGRADLEWGRHRPEEFQLADIFTSAK**KGPIVEFK**LAESTKLVVASCTL -

Fig. 8