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Identification of a cytochrome P450 gene in the earthworm *Eisenia fetida* and its mRNA expression under enrofloxacin stress

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ABSTRACT

Cytochrome P450 (CYP450) enzymes are a family of hemoproteins primarily responsible for detoxification functions. Earthworms have been used as a bioindicator of soil pollution in numerous studies, but no CYP450 gene has so far been cloned. RT-PCR and RACE-PCR were employed to construct and sequence the CYP450 gene DNA from the extracted mRNA in the earthworm *Eisenia fetida*. The cloned gene (EW1) has an open reading frame of 477 bp. The 3′-terminal region contained both the consensus and the signature sequences characteristic of CYP450. It was closely related to the CYP450 gene from the flatworm genus *Opisthorchis felineus* with 87% homology. The predicted structure of the putative protein was 97% homologous to human CYP450 family 27. This gene has been deposited in GenBank (accession no. KM881474). Earthworms (*E. fetida*) were then exposed to 1, 10, 100, and 500 mg kg^{-1} enrofloxacin in soils to explore the mRNA expression by real time qPCR. The effect of enrofloxacin on mRNA expression levels of EW1 exhibited a marked hormesis pattern across the enrofloxacin dose range tested. This is believed to be the first reported CYP450 gene in earthworms, with reference value for molecular studies on detoxification processes in earthworms.

1. Introduction

Cytochromes P450 (CYP450, P450, CYPs) are a super enzymatic family widely distributed in living organisms from bacteria to mammals (Rocha-e-Silva et al., 2001). CYP450 is particularly known for the phase I metabolism of a variety of xenobiotics. Thus these enzymes are of central importance in the detoxification and biotransformation of xenobiotics, and are widely used in many species as biomarkers of pollutants (Lu et al., 2017; Sanchez-Hernandez et al., 2014).

Earthworms are common soil organisms that can be found in most environments and play a crucial role in soil nutrient cycling (Wang et al., 2017). Earthworms also represent one arm of the soil food web in terrestrial ecosystems (Butt and Briones, 2017) and are used in OECD standards as a bioindicator of soil pollution (OECD, 1984). *Eisenia fetida* is the earthworm species most commonly used to survey the quality of the terrestrial environment and has been regarded as a sentinel organism for such studies (Gao et al., 2008). Increasingly molecular approaches are being used in earthworm study to explore the biomolecular information and provide better understanding of how organisms respond to xenobiotics (Conrado et al., 2017; Decaens et al., 2016; Hong et al., 2017).

Current knowledge of CYP450 enzyme activity in earthworms is largely derived indirectly from detoxification studies with specific compounds likely to accumulate as residues in the environment, e.g. the activity measurement of ethoxycoumarin-O-dealkylase (ECOD) (Liimatainen and Haenninen, 1982); benzoxy-resorufin-*O*-dealkylase (BROD) (Achazi et al., 1998; Berghout et al., 1991), Pentoxyresorufin-O-dealkylase (PROD) (Achazi et al., 1998) and Methoxyresorufin-O-deethylase (MROD) (Li et al., 2007; Saint-Denis et al., 1999), as well as CYP450 content and isoenzyme activities (Sanchez-Hernandez et al., 2014; Zhang et al., 2013). The presence of CYP1A2, CYP2E1 and CYP3A4 was recently identified in *E. fetida* (Lu et al., 2017). However, no study that we know of has sequenced a cytochrome P450 gene in an earthworm species.

Across a range of biological organisms CYP genes characteristically contain a conserved sequence: Phe-X-X-Gly-X-X-X-Cys-X-Gly and a "P450-signature": Trp-X-X-X-Arg in their encoded protein (Montellano and Ortiz, 2005). Searching Genbank identified 15 expressed sequence tags (ESTs) associated as putative cytochrome P450 genes of the earthworm *Lumbricus rubellus*. Their GenBank accession no. are EL517373.1, CF810008.1, CF611232.1, DR009436.1, CF610392.1, CO377912.1, CF839202.1, CF610941.1, CV072757.1, CO377912.1, CO058505.1, CF611283.1, CA036213.1, BF422442.2, BF422468.1. On analysis of these ESTs, we did not find the conserved sequence, and the homologies with the reported P450 gene sequences from other organisms were all less than 40% (Montellano and Ortiz, 2005; Nelson et al.,

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2004; Zanger and Schwab, 2013). Therefore, none of these ESTs in Genbank could be confirmed as belonging to the P450 gene.

Given the importance of CYP450 for detoxification in a very wide range of living organisms, and the recognized role of earthworms in maintaining soil health, definition of the CYP450 gene(s) sequence and their functions is desirable, not only to advance understanding of detoxification mechanisms of earthworms and their adaptation to pollutants, but also to generate a suitable knowledge base for future management of various chemical residues within the environment.

Enrofloxacin (EF) is an antibacterial agent used worldwide in veterinary clinical medicine and animal breeding (Martinez et al., 2006), and thus a great amount of EF and its metabolites have been discharged into the environment with animal manure (Li et al., 2016). EF residues in soils cause oxidative stress in earthworm body tissues and increase the toxicity of Cd to earthworms (Gao et al., 2008; Li et al., 2015, 2016). It is known to be metabolized by CYP450 enzymes in animals, and conversely inhibits or induces these enzymes (Al-Nazawi, 2014).

In this study, RT-PCR and RACE-PCR were employed to construct and sequence the CYP450 gene in the earthworm *E. fetida*. Then real time quantitative PCR (qPCR) was used to check the change of mRNA expression of this gene when *E. fetida* were exposed to enrofloxacin in soils. The aims were (i) to screen for candidate CYP450 gene(s) in earthworms, and (ii) to verify the function of the identified gene(s).

2. Materials and methods

2.1. Reagents, soils and animals

Enrofloxacin (99%), Trizol, chloroform and anhydrous alcohol were purchased from Sangon Biotech (Shanghai, China). The RT-PCR kit was purchased from Bio-Rad Laboratories (USA). Avian Myeloblastosis Virus (AMV), Recycling Gel kit, Oligo dT-Adaptor and Taq Polymerase were sourced from Takara (China). All other chemicals and solvents used were analytical grade reagents from commercial suppliers.

Soil from farm land in the Shanghai suburban district, which had not been farmed for more than 20 years, was air-dried after homogenization, and sieved. The soil chemical properties were as follows : pH 7.5, EC 129.5 μS cm^{-1}, organic matter: 32.1 g kg^{-1}, total P: 0.958 g kg^{-1}, total N: 1.85 g kg^{-1}, total K: 2.53 g kg^{-1}. No EF residue was detected in the soil.

Earthworms (*E. fetida*) were purchased from a commercial breeder. They were introduced into the collected soils at least one week before starting the experiment, to adapt to the soil environment. Cow dung with no detectable EF content was dried at 60 °C, ground to pass through a 2 mm sieve, and added as food. Healthy adult earthworms with a well-developed clitellum (0.3–0.5 g) were used for the EF exposure experiments.

2.2. Identification of CYP450 gene in earthworms

2.2.1. Extraction of total RNA

Adult *E.fetida* earthworms as described above were used for RNA extraction, which was carried out using methodology developed from (Wu et al., 2010). After depuration for two days on wet filter paper, the body region anterior to section XXIV was used for RNA extraction as most major organs are located between body sections I and XXIV (Fig. S1) (Bouché, 1972). The selected earthworm body region was then cut into pieces, and 50 mg of tissue finely ground in a mortar, after first immersing in liquid nitrogen and adding 1 mL trizol and 0.2 mL chloroform, centrifuged for 5 min at 12,000 rpm and 4 °C, the supernatant removed, 0.5 mL isopropanol added, the pellet dissolved and then re-centrifuged for 5 min at 12000 rpm (4 °C). The supernatant was discarded, then the residual pellet washed twice with 75% diethylprocarbonate (DEPC, 1 mL) prepared with deionized water; and dried at room temperature before finally being dissolved in 20 μL water and stored at −70 °C. The extracted RNA was tested using 1% agarose gel electrophoresis to make sure that the RNA was not degraded by RNase, and purity was determined by UV spectrophotometry (Agilent Cary 60).

2.2.2. RT-PCR

First-strand cDNA was synthesized with 4 U moloney murine leukemia virus (M-MLV) reverse transcriptase. 1 μg of mRNA and a 50-anchored oligo (dT) 20 primer in 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.4, 200 mM dNTPs) at 42 °C for 20 min. This step was followed by heat-inactivation at 99 °C for 5 min.

The 5′-terminal codons were maximized for the potential of secondary structure formation of the mRNA transcript (Higuchi et al., 1988). A fragment was amplified using primers 5′ -AGATTGTTCTGGT CGCCA-3′ and 5′ -CGGGTTCCTTGGGTCTAACT-3′, which were designed using the primer 5.0 software based on the conservative P450 sequence after comparing, using DNAMAN software, the known CYP450 sequences in Genbank of organisms close to the earthworm in evolution, such as nematodes and schistosomes. The PCR reaction contained 2.5 mM $MgSO_4$, 0.2 μM dNTPs, 200 nM primers, 100 ng of template in a total volume of 100 μL. Each reaction cycle (94 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min) was repeated 38 times and followed by a final elongation step (68 °C for 10 min).

2.2.3. RACE-PCR and sequence determination

The remaining fragment sequences were amplified by RACE-PCR using the primer 5′ -CGGGTTCCTTGGGTCTAACT-3′ and 5′ -GTTTTCCC AGTCACGAC-3′ designed using the primer 5.0 software based on the above results of *RT-PCR* (earthworm P450 gene fragment). The PCR reaction was performed using the same temperature regime as described in Section 2.2.2 above. PCR products were separated by agarose gel electrophoresis and purified using a recycling gel kit, then sequenced by the Beijing Genomics Institute (China).

2.2.4. Sequence analysis

The open reading frame (ORF) was obtained by ORF Finder software (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The gene sequence obtained was compared with CYP450 genes in Genbank from species with evolutionary affinity to the earthworm using Clustal W software (http://www.ebi.ac.uk/Tools/msa/clustalw2/). An unrooted phylogenetic tree was created using the neighbor-joining method by MEGA 7 (http://www.megasoftware.net/) software to show the homology of sequences compared.

2.3. mRNA expression responses of obtained CYP450 gene to stress of enrofloxacin by quantitative real-time PCR

2.3.1. Enrofloxacin exposure experiment

Enrofloxacin was dissolved in DMSO (3 g L^{-1}) and added to milli-Q water for addition to soils, to form five concentration groups of 0, 1, 10, 100, 500 mg per kg dry soils respectively, with four replicates for each group. The soil concentrations of EF reach 1 mg kg^{-1} in grazed areas, and sometimes as high as 10 mg kg^{-1} soon after the application of animal manure (Tai et al., 2012; Zhao et al., 2010). The 100 and 500 mg kg^{-1} concentration treatments were included in order to observe the toxicological response at a high dose exposure. Such concentrations might occur following accidental spillage or deliberate illegal discharge of farm or pharmaceutical factory wastewater, or farm wastes such as poultry litter with high EF concentration. After spiking of EF, the treated soils were mixed well and placed in glass jars (1.5 kg soil per jar; in total 20 jars for the five concentration groups). Then the jars were kept in a laboratory draught cupboard and turned regularly for two days to allow DMSO to evaporate. Soil moisture content in all treatments was adjusted to 35% (w/w) with distilled water.

Twenty earthworms were washed quickly, and added into each glass jar one week after EF spiking. The jars were then placed in a dark room at 20 °C. Cow dung was added to the soil surface (2 g per week in each jar) to feed the earthworms. The jars were weighed two times per week