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Short communication

# Differential expression of genes in the earthworm *Eisenia fetida* following exposure to *Escherichia coli* O157:H7

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ABSTRACT

In this study, suppression subtractive hybridization was used to construct forward and reverse cDNA libraries to identify genes involved in the response of *Eisenia fetida* after exposure to *Escherichia coli* O157:H7. We cloned 1428 cDNAs or expressed sequence tags (ESTs), of which 738 were confirmed to be differentially expressed on dot blotting analysis. A total of 394 good-quality ESTs (GenBank dbEST accession numbers HO001170–HO001563) were obtained from the raw clone sequences after cleaning. The genes were associated with metabolism (10%), transport (10%), translation (5%), immunity (2%), and the cytoskeleton (1%). Thirteen candidates were selected to assess expression levels in earthworms exposed to artificially contaminated soil by real-time PCR. The translated amino acid sequences of clones were similar to fibrinolytic protease 1, extracellular globin-3, myosin essential light chain, lumbrokinase, lysozyme, ferritin, ATP synthase F0 subunit 6, and hsp 70. Characterization of differential gene expression in the earthworm *E. fetida* on exposure to *E. coli* O157:H7 expands our understanding of the molecular mechanisms of interactions at the earthworm–pathogen interface.



## 1. Introduction

The bacterium *Escherichia coli* O157:H7 is a foodborne pathogen that has been implicated in many outbreaks of infectious diseases worldwide. It may cause hemorrhagic colitis, hemolytic-uremic syndrome, thrombotic thrombocytopenic purpura, and even death (Tarr, 1995). Infected animals typically excrete more than $10^8$ colony-forming units (CFU) of *E. coli* O157:H7 per gram of feces, and virulent *E. coli* strains can survive for several months in animal waste (Fukushima and Seki, 2004). However, *E. coli* O157:H7, as a toxin-producing food- and waterborne bacterial pathogen, has been linked to large outbreaks of gastrointestinal illnesses in humans for more than two decades (Manning et al., 2008). Thus, effective and convenient methods to reduce or eliminate pathogens in organic manure are desirable and would benefit human health.

The Annelida Oligochaeta group plays a key role in most continental ecosystems, and represents an important part of the soil macrofauna (Brulle et al., 2008). As a key representative of soil fauna, earthworms are essential in maintaining soil fertility through their burrowing, ingestion, and excretion (Edwards, 2004). Earthworms are increasingly recognized as indicators of agroecosystem health and an ecotoxicological sentinel species because they are constantly exposed to soil contaminants (Pirooznia et al., 2007). Despite the absence of an adaptive immune system, based on antibodies or lymphocytes, invertebrates have evolved various active defense pathways that efficiently recognize and respond to non-self substances (Köhlerová et al., 2004). The innate immune system of invertebrates includes many reactions, with encapsulation being a critical component, providing an effective obstacle to infection alongside phagocytosis and antimicrobial peptides (Field et al., 2004). In the course of evolution, earthworms have developed efficient defense mechanisms against microbes ingested during feeding or taken up into the body from the environment after injury (Cooper and Roch, 2004). Earthworms are considered one of the most suitable representatives of soil animals for soil contamination surveys (Reinecke and Reinecke, 2004). Gene expression profiles represent the first level of integration between environmental stressors and the genome, which pilots the response of the organisms to external changes via the synthesis of proteins (Brulle et al., 2008). Due to their ecological importance, extensive genomics studies have been performed in earthworms as test organisms in terrestrial ecotoxicology (Pirooznia et al., 2007; Stürzenbaum et al., 1998; Owen et al., 2008; Svendsen et al., 2008). In the absence of full genome sequence data, expressed sequence tags (ESTs) allow for the rapid identification of expressed genes by sequence analysis, and are thus an important resource for comparative and functional genomic studies. Several EST projects have been performed in *Lubricus rubellus* (Stürzenbaum et al., 2003), *Eisenia andrei* (Lee et al., 2005), and *Eisenia fetida* (Pirooznia et al., 2007), which have generated 19,934, 1108, and 4032 ESTs, respectively. To understand

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the gene expression response to *E. coli* O157:H7 contaminants, we cloned and sequenced putative differentially expressed fragments obtained by suppressive subtractive hybridization-PCR (SSH) and further confirmed their expression patterns using reverse Northern blotting analysis.

This work was performed as part of a larger effort to identify candidate molecular biomarkers for rapid, mechanism-based gene expression assays to supplement current acute and reproductive toxicity tests. The specific objectives of this study were to isolate and characterize cDNAs from *E. fetida* that could be used to monitor exposure to *E. coli* O157:H7.

## 2. Materials and methods

### 2.1. Animals and treatment

#### 2.1.1. Experimental organism and preparation of artificial soil

Adult *E. fetida* weighing 300–400 mg were purchased from the earthworm farm of China Agricultural University and kept in test substrates for 1 week before the experiment. *E. fetida* corresponds to the striped or banded morph, with the area around the intersegmental groove having no pigmentation and appearing pale or yellow, hence its common names of "brandling" or "tiger" earthworm (Reinecke and Viljoen, 1991). Artificial substrates were prepared as described in the OECD (OECD, 1984) guidelines. Food was first provided with 0.5% air-dried cow manure and then provided every 14 days for the longest exposure periods; $10^7$ CFU $g^{-1}$ soil (colony forming units per gram of dry matter) of O157:H7 was added to the soil within half an hour of introducing the worms. Concentrations of O157:H7 were reported for *E. fetida* in OECD artificial soil (Liu et al., 2009; Wang et al., 2010). For the SSH, three groups of 25 adult worms were used per condition (75 worms per condition) and exposure lasted 3 or 30 days.

The international reference strain of enterohemorrhagic *E. coli* O157:H7 EDL933 was purchased from the Institute of Epidemiology and Microbiology, Chinese Academy of Preventive Medicine. *E. coli* O157:H7 is a foodborne pathogen. Infection routes of pathogenic *E. coli* O157:H7 are food and water into the intestine. We wore masks and medical latex gloves in the experiment. After each experiment, all objects that came into contact with the virulent *E. coli* O157:H7 were cleaned with antiseptic solution (benzalkonium bromide), and all experimental equipment was sterilized at 121 °C for 20 min.

### 2.2. RNA isolation and purification

At the end of the *E. coli* O157:H7 exposure, groups of 25 earthworms were removed quickly, snap frozen in liquid $N_2$, and stored at −80 °C until use. Total RNA was extracted from the collected whole worm samples using the TRIzol® Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. After deoxyribonuclease I treatment, quantity and purity of RNA samples were assessed using ultraviolet spectrophotometry; the integrity was verified by electrophoresis on 1% agarose gels stained with ethidium bromide. The RNA pellets were stored at −80 °C until needed.

### 2.3. Suppression subtractive hybridization

To optimize the detection of transcripts regulated by exposure to *E. coli* O157:H7, subtractive libraries were made using four pooled samples: three groups of 25 unexposed adult worms on day 3, three groups of 25 exposed adult worms on day 3, three groups of 25 unexposed adult worms on day 30, and three groups of 25 exposed adult worms on day 30. Aliquots of 250 mg of total RNA from each group were used to prepare 2 mg of mRNA (exposed and control). mRNA was isolated from total RNA using an Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany). Products of the first and second strand cDNA synthesis were digested with the endonuclease *Rsa*I. After ligation and hybridization, PCR amplification was performed as described in the PCRselect cDNA subtraction manual (Clontech, Palo Alto, CA). PCR products were cloned using a TA Cloning Kit for Sequencing (Promega, Madison, WI) with the pGEM-T Easy vector and transformation-competent *E. coli*, strain DH5σ.

Insert amplification was performed by randomly picking individual colonies and adding them directly into the PCR mix. PCR was done with Taq polymerase (TaKaRa, Kyoto, Japan) in 96-well plates using nested primers and typical PCR cycling conditions: 2.5 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C.

### 2.4. Reverse Northern blotting analysis

To eliminate false-positive results, we used reverse Northern blotting. The probes were labeled with a DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, Mannheim, Germany) in accordance with the manufacturer's instructions. The membranes were then subjected to hybridization with DIG-labeled fragments and immunological detection according to the manufacturer's instructions. Results were documented by photocopying the wet filters. Both membranes were used to identify the background in control versus induced samples. The fold-induction of dot densitometry was qualified using Dot Blot Analyzer software (Sage Creation, Beijing, China). *E. fetida* metallothionein (MT) and β-actin cDNAs were used as positive and loading controls, respectively. The blotting experiments were repeated three times.

Unique sequences from contigs or singletons were used for database searches with BLASTX and BLASTN (http://www.blast.ncbi.nlm.nih.gov/BLAST.cgi). Gene function was inferred from homology to sequences in GenBank and the Gene Ontology classification (http://www.geneontology.org/).

### 2.5. Quantitative real-time PCR analysis

Analysis of differential gene expression was performed by SYBR qRT-PCR. The extraction was repeated three times for each sample (three biological replicates). The samples were treated with RNase-free DNase I (TaKaRa Biotechnology, Dalian, China) to remove any contaminating DNA. After this step, we checked the purity and integrity of RNA by electrophoresis on 1% agarose gels stained with ethidium bromide. We then checked the purity of RNA by PCR without reverse transcription using β-actin gene-specific primers.

Aliquots of 2 mL of each RNA sample were used to construct cDNAs using the PrimeScript™ RT reagent kit (Perfect Real Time; TaKaRa Biotechnology). Primer pairs were designed using the Primer Express 3.0 software (Table 1S, Supplementary data). Gene expression was assayed using the ABI PRISM 7900HT Fast and Quantitative. Polymerase chain reaction (qPCR) was performed using SYBR® Premix Ex Taq™ II (Perfect Real Time; TaKaRa Biotechnology) with a two-step reaction. Results are expressed as the normalized ratio of the mRNA level of each gene of interest to that of the housekeeping gene using the difference between threshold cycle values or the $C_t$ method (Livak and Schmittgen, 2001). $C_t$ values for individual target genes were calculated, and the $C_t$ average for the housekeeping gene (β-actin mRNA) was treated as an arbitrary constant and used to calculate $C_t$ values for all samples. The fold induction, resulting in three independent pools for each target gene, was averaged and the SEM was calculated.