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Identification and expression profile of gene transcripts differentially expressed during metallic exposure in *Eisenia fetida* coelomocytes

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Summary

The aim of this work was to identify in *Eisenia fetida* genes whose expression are regulated following exposure to a complex mixture of metallic trace elements (MTE) representative of a highly polluted smelter soil. Suppression subtractive hybridization (SSH) was used to construct cDNA libraries enriched in up- or down-regulated transcripts in the immune-circulating cells of the coelomic cavities, namely coelomocytes, from worms exposed to metallic pollution. Among 1536 SSH-derived cDNA clones sequenced, we identified 764 unique ESTs of which we selected 18 candidates on the basis of their redundancy. These selected candidates were subjected to a two-step validation procedure based on the study of their expression level by real-time PCR. The first step consisted in measuring the expression of the 18 candidates in worms exposed to artificial contaminated soil. The second step consisted in measuring the expression in animals exposed to a “naturally” contaminated soil sampled close to a smelter. Both steps allowed us to highlight 3 candidates that are strongly induced in worms exposed to a smelter polluted soil. These candidates are: the well-known MTE-induced Cd–metallothionein and 2 original

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biomarkers, lysenin, and a transcript, which cloning of the complete coding sequence identified as the coactosin-like protein.

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Introduction

Human activities, especially metallurgical ones, lead to accumulation of metal trace elements (MTE) in the topsoils, where one typically observes concentrations largely exceeding the natural background. Heavy contaminations of soils are a threat to public health. Cases of cancer related to high cadmium concentrations have been described [1].

Moreover, the presence of high amounts of these MTE constitutes a major stress likely to disturb the reproduction and immunity functions of animals [2]. It has been also demonstrated that metallic pollution sensitizes invertebrates used in aquaculture (mussel, oyster, shrimp) to microbial infections by altering the defence processes [3,4]. MTE can generate an environmental stress likely to affect the organisms exposed to polluted soils. Plants and animals living in close contact with polluted soils were studied in order to better understand the physiological changes, the mechanisms of acclimation and the mechanisms of detoxification caused by metals [5,6]. According to Weeks [7], a “biomarker” is a biological response to a chemical (pesticides, polycyclic aromatic hydrocarbons, MTE, ...), which induces a stress and could assess exposure or point out toxic effects. Biomarkers have been also defined as any observable and/or measurable variation at the molecular, biochemical, cytological, physiological, biological or behavioural level revealing past or present exposure of an organism to one or several pollutants [8]. Most are early markers of exposure that do not reveal long-term effects on the ecosystem. The development of biomarkers allowing an early diagnosis of long-term ecosystem dysfunctions represents a challenge for ecotoxicologists. The emergence of molecular biology techniques applied to ecotoxicology, allowed a better understanding of the mechanisms of action of contaminants in living organisms. Indeed, gene expression profiles represent the first level of integration between environmental stressors and the genome, which, through the synthesis of proteins, pilots the response of the organisms to external changes. Thus, analysis of the changes of gene expression is a powerful tool, (1) to diagnose the existence of a stress in a population and (2) to analyse mechanistically the response to a stress.

Soil health and sustainable productivity depends on living organisms, which affect the cycling rate and availability of the major organic and non-organic compounds in their constant search for food and energy sources [9]. One of the most studied faunistic groups is the Annelida Oligochaeta group. These animals play a key role in most continental ecosystems, represent an important part of soil macrofauna and are implied in the maintenance of the structure and the fertility of soils. Immunotoxicological studies demonstrated that MTE can affect phagocytic activity in *Lumbricus terrestris* [10] and in *Eisenia andrei* [11]. Annelids are usually strongly affected by a metal pollution. For example,

metallic pollution of the soil can delay sexual maturation [12], slow down growth [13], modify enzymatic activities [14], and modify gene expression [15–17]. For 10 years, analysis of gene expression profiles has made it possible to identify biomarker candidates in Oligochaeta Annelida. The best known candidate is the metallothionein (MT), a protein of low molecular weight (6000–8000 Da), rich in cysteines (approximately 30%), involved in detoxification of metals such as cadmium and in the homeostasis of essential trace elements such as zinc [18,19]. MT is regarded as a good biomarker of exposure because it shows a dose and time-dependent increase of the protein and of the number of transcripts coding MT when worms are exposed to a metal contamination and especially cadmium [15,20].

In a recent study, we partially cloned and measured the gene expression of 14 potential biomarkers in the laboratory model *Eisenia fetida* after *in vivo* exposure to Cd in artificial soil. Effectors were chosen among highly preserved proteins for which variations (in terms of protein quantity and/or expression) following metal exposure were reported in the literature. Expression was measured in coelomocytes since the majority of the selected effectors were known to be involved in defence mechanisms [15]. However, this “targeted” approach provided no better candidate than MT. Thus, we undertook an un-selective strategy by producing SSH libraries on the same model. In the present study, this molecular biology technique was used to identify transcripts exhibiting variations of their amount following metal exposure. Worms were exposed to artificial soil contaminated by a realistic mixture of MTE i.e. metal concentrations, which can be found in a strongly polluted smelter soil. The aim of the study being the identification of genes, which response early and consistently across timepoints, kinetics of intoxication of 1 and 14 days were performed. Biomarker candidates selected after SSH, were submitted to two validation steps. The first step consisted in the analysis of the expression level of these candidates using real-time PCR in animals exposed to contaminated artificial soil. The second step of validation was an analysis of the expression level of the candidates validated in step 1 in animals exposed to a “naturally” polluted soil originating from Metaleurop, the main European lead smelter located in the north of France that generated significant atmospheric emissions (in 2001, Pb: 18.4 tons, Zn: 26.2 tons, Cd: 823 kg, [21]) until 2003, the closing date of its activities.

Material and methods

Animals and treatment

Adult earthworms came from controlled cultures in laboratory. They were bred at an ambient temperature of 22 ± 2 °C, in the dark, on vegetable mould with fresh cattle manure as food source. Moisture content was maintained

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