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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.

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 $22 \pm 2\,^{\circ}C$, in the dark, on vegetable mould with fresh cattle manure as food source. Moisture content was maintained

around 60%. Artificial substrates were prepared as described in the OECD guidelines [22]. Briefly, the substrate was composed (percentages refer to dry weight) of 10% sphagnum, 20% kaolinite clay, and 70% quartz sand. The pH was adjusted to 6 ± 0.5 with $CaCO_3$. Metals ($CdCl_2$, $PbCl_2$ and $ZnCl_2$) were added to the soil within half an hour of introducing the worms. The concentrations used were 40 mg kg^{-1} of Cd, 500 mg kg^{-1} of Pb and 700 mg kg^{-1} of Zn. For the SSH, three groups of 25 adult worms were used per condition (75 worms per condition) and exposure lasted either 1 or 14 days. At the end of the exposure periods, worms were rinsed in distilled water, coelomocytes were extruded and cellular RNA was extracted as described below.

At the same time, the field soil was collected from a site located near the former smelter. Soil was collected from the top 20 cm layer after removal of vegetation, transferred to the laboratory and fresh soil aggregates were broken up then air-dried and sieved through a 4 mm mesh. Physical–chemical parameters of soil are presented in Supplementary Table 1. Total Cd, Pb and Zn and $CaCl_2$ extractible concentrations were measured in artificial and in Metaleurop soils according to previously described procedures [23,24].

Real-time PCR analysis was done using worms (pools of 25 animals) exposed 1 or 14 days to metal-spiked artificial soil (like the worms used for the SSH) or exposed to a naturally contaminated soil from Metaleurop.

Ethanol and electrical extrusion

Groups of 25 worms were placed for 5 min in a 50-mL polypropylene tube containing 30 mL of cold extrusion medium (2.5 mg mL^{-1} EDTA, 8.5 mg mL^{-1} NaCl, pH 7.3, 5% ethanol). Then, worms were stimulated 10 times using short (<5 s) exposure to 9 V alternate current [25]. After removal of the worms, a centrifugation (2000*g*, 6 min) was performed to collect pellets.

Suppression subtractive hybridization

In order to optimize detection of transcripts regulated after contamination, the subtractive libraries were made using four pooled samples: 3 groups of 25 uncontaminated adult worms at 1 day, 3 groups of 25 uncontaminated adult worms at 14 days, 3 groups of 25 contaminated adult worms at 1 day and 3 groups of 25 contaminated adult worms at 14 days. Coelomocytes total RNA was extracted from each group using Tri-Reagent (Molecular Research Center Inc., Cincinnati, USA) according to manufacturer's instructions. One hundred micrograms of total RNA from each group were used to prepare 2 μg of mRNA (contaminated and control). mRNA were isolated using polyA Spin mRNA Isolation Kit (New England BioLabs Inc., Ipswich, MA, USA). The products of first and second strand cDNA synthesis were RsaI endonuclease digested. After ligation and hybridization, PCR amplifications were performed as described in the PCR-select cDNA subtraction manual (Clontech, Palo Alto, CA, USA). PCR products were cloned using TOPO TA Cloning Kit for Sequencing (Invitrogen Life Technologies, France) with the pCR4-TOPO vector and chemically competent TOP10F' One Shot *Escherichia coli*.

Insert amplification was performed by picking randomly individual colonies and putting them directly in PCR mix. PCR reactions were done with GoTaq polymerase (Promega) in 96-wells plates using M13 primers and typical PCR cycling conditions: 10 min at 94 °C then 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C for 25 cycles. PCR products were purified in 96-wells plates using UltraClean-htp™ 96 Well PCR Clean-Up Kit (MoBio Laboratories Inc., Carlsbad, USA) according to manufacturer's instructions. cDNA fragments isolated from the individual clones were sequenced using a dideoxydye-terminator method (CEQTM DTCS-Quick Start kit, Beckman coulter) and a CEQTM 8000 apparatus (Beckman Coulter). Sequencing was performed using the forward primer T7 (TAATACGACTCACTATAGGG). Sequences were analysed using the Sequencher™ software (Gene Codes Corporation). Unique sequences (from contigs or singletons) were submitted to database searches using BLASTX and BLASTN softwares (http://blast.genome.jp/ or http://www.ncbi.nlm.nih.gov/BLAST/). Specific domain searches were performed using RPS-BLAST (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Gene function was inferred from sequence identity with Genbank database and the "Gene Ontology" classification (http://www.geneontology.org/).

Quantitative RT-PCR

Total RNA from pools of 25 individuals from each treatment (control or contaminated after 1 or 14 days of exposure) were used. Specific primers for real-time quantitative PCR (Supplementary Table 2) were edited using the LightCycler Probe Design software version 1.0 (Roche Diagnostics). Levels of expression and amplification efficiencies (*E*) of each target gene (Tg) were determined according to previously described procedures [15]. For each sample, the levels of expression of the target gene (Tg) were compared with the expression of the constitutively expressed β-actin (act) gene and MT gene expression was used as a positive control for metallic exposure [15,16]. The expression ratio (*R*) was calculated according to the formula $R = (E_{Tg})^{CPTg}/(E_{act})^{CPact}$. Another constitutively expressed gene coding the ribosomal protein S13 was used as an additional control. In order to simplify data presentation, relative expression ratios of Tg's are presented (expression ratios in exposed earthworms relative to expression ratios in control unexposed earthworms).

Rapid amplification of cDNA ends (RACE)

In order to get the full-length cDNA of genes with unknown function but exhibiting high variations of their transcript levels, 5′RACE was performed according to a previously described procedure [26].

Metal analysis

Cd, lead (Pb) and zinc (Zn) contents were determined by inductively coupled plasma optical atomic emission spectroscopy (ICP-AES) (Varian Liberty Serie II axial view, Varian S.A., Les Ulis, France) (adapted from [27]). Calibration was done with standards for each metal (0, 0.01, 0.05, 0.1, 0.5,

1 mg Cd L^{-1} or 0; 0.05; 0.1; 0.2; 0.4; 0.6 mg Pb L^{-1} or 0; 0.1; 0.25; 0.5; 1; 2 mg Zn L^{-1}) prepared in 500 mmol L^{-1} nitric acid (65% Suprapur, VWR, Fontenay-sous-Bois, France.) with a commercial standard stock solution of 1 g L^{-1} of Cd, Pb and Zn Titrisol (VWR). Cd, Pb and Zn contents in whole worms (3 groups of 5 animals per condition) were determined after acid digestion. Briefly, worms were lyophilised for 3 days then digested for 12 h at room temperature with 1 mL of 65% nitric acid per 100 mg (dry mass). Digestion was performed by progressive heating at 120 °C until half reduction of the volume and nitrous vapour removal. After cooling, 1 mL of a solution of analytical grade nitric, sulphuric and perchloric acids (10, 2, 3 V, respectively) was added to samples and heating was followed at 180 °C for reducing the volume. Samples were then filtered and volume was adjusted to 20 mL with ultrapure water (Milli-Q water, Millipore, Molsheim, France).

Statistical analysis

Expression levels were compared by means of two-way analysis of variance followed by Student post-hoc *t*-tests. The normality of each distribution was verified by Kolmogorov–Smirnov's tests and variance heterogeneity by Hartley's tests.

Metal contents were analysed using Kruskal–Wallis non parametric test followed by Mann–Whitney test. An α-level of 0.05 was used in all procedures.

Results

EST sequencing and general characteristics of SSH library

A total of 1536 clones from exposed SSH libraries (''forward'' libraries, i.e., subtracted with control) and from control (''reverse'' library, i.e., substracted with exposed) were sequenced. Contaminant sequences corresponding to vector and adaptator were removed and redundant ESTs were organized into overlapping contigs using the Sequencher™ 4.2 software (Gene Codes Corporation). A total of 764 unique sequences from forward (1 and 14 days grouped) and reverse libraries (Genbank accession numbers EY892395–EY893158) were tested to detect potential alignment of ESTs sequenced and discard false positive SSH–ESTs (for details see Table 1).

Identification of candidate ESTs

ESTs were confronted to databases using BLASTN, BLASTX and RPS-BLAST programs [28]. Sequences similarities were considered to be significant when the expected values were less than 10^{-3}. Comparisons of ESTs against non-redondant nucleic and protein databases revealed that 48.04% of the ESTs did not align significantly with known genes, and 24.34% align with genes of unknown function. Among the remaining 27.62%, ESTs presented significant similarities with genes of known function. These 211 ESTs were assigned to function based on their sequences identity with Genbank™ database, RPS-BLAST and according to ''Gene Ontology'' classification. Thus, 211 ESTs were associated to six major cellular physiological functions, (i) immunity; (ii) metabolism; (iii) signal transduction; (iv) replication, repair, transcription and translation; (v) cytoskeleton production and maintenance; and finally (vi) transport (see Figure 1 and Table 1 for library by library details).

In order to identify cDNAs corresponding to *E. fetida* genes regulated by metals, we chose 18 candidates, which showed the highest abundance (redundant clones) (Table 2). Candidate 1 showed similarity with lysenin (Lys), a protein involved in immunity. This protein is produced in large coelomocytes and free large chloragocytes [29]. Identified

Table 1 General characteristics of 3 SSH libraries from *Eisenia fetida* exposed to metal-spiked soil.

	Forward 1 day	Forward 14 days	Reverse 14 days
Total numbers of cDNAs sequenced	384	768	384
Total numbers of cDNAs analysed[a]	262	558	265
Singletons	198	273	142
EST contig	35	258	94
Redundancy[b] (%)	13.36	46.24	35.47
Average EST length (for three SSH libraries)	305 bp		
Functional categories			
No hits (%)	53.27	45.31	47.46
Unknown (%)	16.36	28.42	25.42
Immunity (%)	0.93	3.75	3.39
Metabolism (%)	18.22	12.33	10.17
Signal transduction (%)	3.74	2.14	2.82
Replication, repair, transcription, translation (%)	4.67	5.36	5.08
Cytoskeleton production and maintenance (%)	1.40	2.14	4.52
Transport (%)	1.40	0.54	1.13

EST, expressed sequenced tags.
[a]Sequences used to interrogate databases using Blast software after analysis using Sequencher™ 4.2 software.
[b]Redundancy = number of ESTs assembled in contig/total ETSs.

in *E. fetida*, the lysenin is a haemolytic protein, which binds specifically to sphingomyelin and forms oligomers, leading to the formation of pores with a diameter of ~3 nm in target membranes [30–32]. Lysenin is closely related to fetidin. The name lysenin was chosen because of the *E*-value given by the Blast analysis (*E*-value of 0 and of 5e−141, respectively with Blast N and Blast X).

Candidates 2–6 were similar to proteins involved in metabolism. As expected, the Cd-metallothionein (Cd-MT, candidate 2) was among these 5 candidates as one of the most abundant transcripts ($n = 10$) sequenced in both forward SSH libraries (1 and 14 days of exposure). Candidate 3 was similar to the Cytochrome c oxidase subunit 1 (CytOx c). This gene provides a critical function during respiration by transferring electrons from Cytochrome c to oxygen and contributing to ATP generation [33]. Candidate 4 was similar to cytochrome b (Cyto b) from various species, a component of the ubiquinol–cytochrome c reductase complex (complex III or cytochrome b–c1 complex), which is a respiratory chain that generates an electrochemical potential coupled to ATP synthesis. Candidate 5 was similar to the translationally controlled tumor protein (TCTP). TCTP is induced in *Lumbricus rubellus* by metallic exposure [34]. Candidate 6 was the last putative metabolism protein and exhibited similarity to granulin (Granu). Granulin belongs to a family of cysteine-rich peptides of about 6 kDa derived from a larger precursor. Both have been found to modulate cell growth [35].

Two candidates were similar to proteins involved in signal transduction. Candidate 7 was similar to a sarcoplasmic calcium-binding protein (SCBPs). SCPs are an important Ca^{2+}-binding system in muscles of both vertebrate and invertebrate species. The SCP from the sandworm *Nereis diversicolor* is a single polypeptide chain of 174 amino acids and a molecular weight of 19,485 Da. There are three calcium-binding sites, which may be occupied by magnesium ions under physiological conditions [36]. Candidate 8 was similar to parvalbumin (Parvalb). The protein encoded by this gene is a high-affinity calcium ion-binding protein believed to act as cytosolic Ca^{2+} buffers [37] that is structurally and functionally similar to calmodulin and troponin C. The unique sequence 9 was similar to ribosomal protein S16 (Ribo S16), which is involved in translation.

Four candidates were similar to proteins with unknown functions. Candidate 10, 11 and 12 aligned with ESTs from different *L. rubellus* ESTs libraries, whereas candidate 13 was similar to a predicted protein of *Nematostella vectensis*.

Finally, candidates 14–18 were unknown proteins with unknown functions (No Blast Hit).

Expression profiles in artificial soils

Expression levels of the 18 selected EST candidates were measured using real-time PCR in worms exposed to either uncontaminated (control) or metal-polluted (exposed) artificial soil.

This first step of validation was done with RNA samples from a second exposure experiment in order to eliminate candidates potentially regulated differentially between experiments.

The vast majority of the chosen candidates (14 out of the 18 analysed) showed moderate to strong variations in transcript levels associated with exposure to metals (Figure 2 and Table 3). As expected, transcript levels of the Cd–MT were strongly increased although this increase was only detectable after 14 days of exposure to the metallic

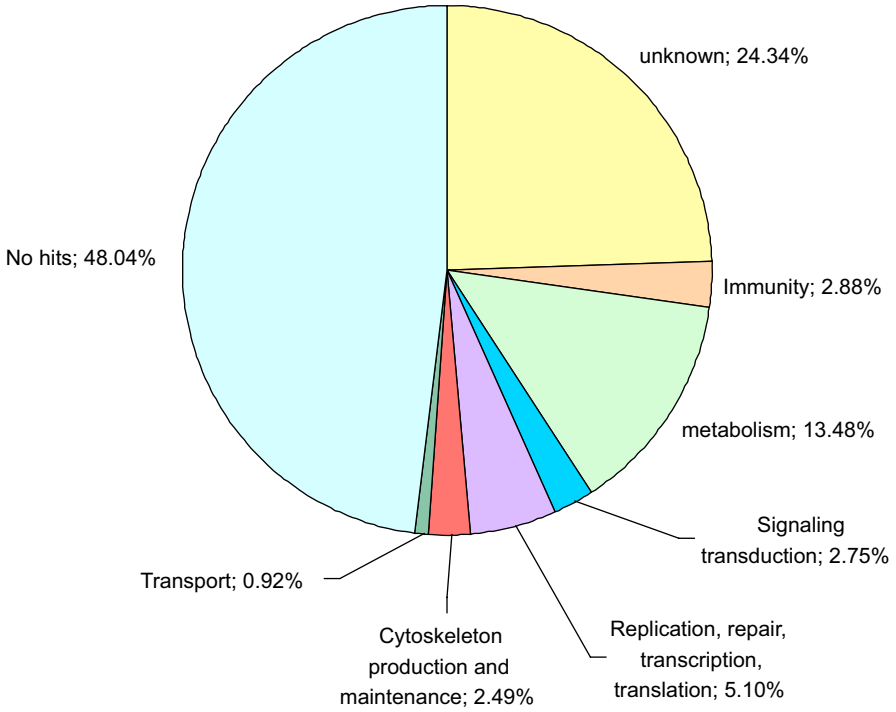

Figure 1 Number of differentially expressed genes (%) in each gene category after submission to Blast databases and according to "Gene Ontology" classification (http://www.geneontology.org/).

Table 2 Characteristics of the 18 SSH-EST candidates selected from the exposed (*E*) or control (Ctl) libraries of *E. fetida*.

Unique sequences	Accession number (name)	Length of longest or consensus fragment-bp-(number of clones)	Blast similarity/sps/ accession on no	Best blast *E*-value	RPS-Blast (*E*-value)	Domain function
1 (*E*)	EY892971 (Lys)	828 ($n = 6$)	Lysenin/*Eisenia fetida*/BAA21518 [a]	5e−141	None	
2 (*E*)	EY892898 (Cd-MT)	502 ($n = 10$)	Cd-metallothionein/ *Eisenia fetida*/ P81695 [a]	4e−21	None	
3 (*E*)	EY892966 (CytOx c)	559 ($n = 4$)	Cytochrome c oxidase subunit I/ *Eisenia fetida*/ ABM66354 [a]	5e−69	cd01663/ Cyt_c_Oxidase_I/ (2e−86)	Terminal oxidase in the respiratory chains of eukaryotes. Catalyze the reduction of O_2 and simultaneously pumps protons across the membrane of mitochondria
4 (*E*)	EY892965 (Cyto b)	332 ($n = 2$)	Cytochrome b/ *Lumbricus terrestris*/ NP_008243 [a]	8e−50	cd00284/ Cytochrome_b_N/(3e−21)	Cytochrome b is a subunit of cytochrome bc1, an 11-subunit mitochondrial respiratory enzyme. Contains two bound hemes and two ubiquinol/ubiquinone binding sites
5 (*E*)	EY892907 (TCTP)	493 ($n = 2$)	TCTP/*Lumbricus rubellus*/O18477 [a]	3e−65	pfam00838/ Translationally controlled tumour protein/ (4e−22)	
6 (*E*)	EY892886 (Granu)	552 ($n = 3$)	Granulin/*Mus musculus*/ EDL34132 [a]	1e−20	smart00277/ Granulin/ (6e−04)	
7 (*E*)	EY892976 (SCBP)	415 ($n = 3$)	SCBP2 protein/ *Lumbricus terrestris*/ CAE18114 [a]	1e−08	cd00051/EF-hand, calcium binding motif/ (2e−04)	A diverse superfamily of calcium sensors and calcium signal modulators; Ca^{2+} binding induces a conformational change in the EF-hand motif, leading to the activation or inactivation of target proteins
8 (*E*)	EY892890 (Parvalb)	434 ($n = 3$)	MGC107867 protein (similar to parvalbumin)/ *Xenopus tropicalis*/ NP_001015721 [a]	2e−04	cd00051/EF-hand, calcium binding motif/ (2e−04)	Same as above
9 (*E*)	EY892967 (RiboS16)	462 ($n = 4$)	16S ribosomal RNA gene/*Eisenia fetida*/ EF126208 [b]	9e−169	None	
10 (*E*)	EY892879 (Cand10)	402 ($n = 5$)	Earthworm Copper Exposure Library/ *Lumbricus rubellus*/ DR009691 [b]	2e−16	None	

Table 2 (*continued*)

Unique sequences	Accession number (name)	Length of longest or consensus fragment-bp-(number of clones)	Blast similarity/sps/accession on no	Best blast *E*-value	RPS-Blast (*E*-value)	Domain function
11 (*E*)	EY892881 (Cand11)	472 ($n = 5$)	Juvenile Earthworm Library/*Lumbricus rubellus*/CO869889 [b]	5e−38	None	
12 (*E*)	EY892889 (Cand12)	415 ($n = 3$)	Earthworm Head Enriched library/*lumbricus rubellus*/CO378146 [b]	5e−05	None	
13 (Ctl)	EY893141 (Cand13)	284 ($n = 4$)	Predicted protein/*Nematostella vectensis*/XP_001638172 [b]	5e−31	None	
14 (*E*)	EY892888 (Cand14)	396 ($n = 3$)	No Blast Hit		None	
15 (*E*)	EY892892 (Cand15)	318 ($n = 3$)	No Blast Hit		None	
16 (*E*)	EY892903 (Cand16)	555 ($n = 4$)	No Blast Hit		None	
17 (*E*)	EY892882 (Cand17)	399 ($n = 5$)	No Blast Hit		None	
18 (*E*)	EY892908 (Cand18)	739 ($n = 6$)	No Blast Hit		None	

[a]BlastX results.
[b]BlastN results.

mixture (5.75 fold induction and $p = 0.001$). According to the SSH results, both TCTP and candidate 10 were expected to be up-regulated by metallic exposure. Accordingly, they were characterized by a moderate but significant induction of their expression, after 1 and 14 days of exposure for TCTP (1.81, $p = 0.031$ and 2.11 fold, $p = 6.10^{-4}$, respectively) and after 14 days of exposure for the candidate 10 (2.34 fold and $p = 0.005$). Thus, the real-time PCR analysis confirmed this up-regulation.

A group of 3 candidates showed a 3 fold induction of their transcript levels. These were the Cyto b, the Ribo S16 and the candidate 12. Candidates from these groups were expected to be up-regulated, which is consistent with the observed expression profiles.

Three other candidates, namely Lys, candidates 15 and 17, displayed the highest variations of their expression ratios. This group is characterized by a strong and significant induction (5.89–21.84 fold) of each gene.

Finally, a last group contains 5 candidates exhibiting variation of expression they were not expected to. Indeed, CytOx c, SCBP, Parvalb and candidate 18 showed a substantial decrease of their levels of expression whereas they were expected to be up-regulated. Similarly, candidate 13 showed a significant increase of its transcript levels whereas it was expected to be down-regulated. Expression levels of these genes were not measured in animals exposed in ''naturally'' polluted soils.

Transcript levels of only 4 candidates (Granu, candidates 11, 14 and 16) did not vary substantially.

Overall, of the 14 candidates showing variations of transcript levels, we confirmed 9 of them had the expected increase in transcript abundance.

Expression profiles in a field soil

In a second step of validation, expression levels of the 9 remaining candidates (validated in step 1) were measured in animals exposed to a contaminated soil originating from Metaleurop for either 1 or 14 days. Expression levels of 6 candidates (Cyto b, TCTP, Ribo S16, candidates 10, 12 and 15) did not show significant variation in response to the metallic stress. Three candidates exhibited significant increase of their level of expression (Figure 3 and Table 4).

Cd–MT showed a significant increase of its transcript levels after 14 days of exposure to the field soil (10.4 fold and $p = 0.041$).

Lys was characterized by an induction of its expression for all exposure durations (1.42 fold and $p = 0.031$ after 1 day of exposure and 2.76 fold and $p = 0.012$ after 14 days of exposure).

Candidate 17 showed a significant increase of the quantity of mRNA expressed after 1 and 14 days of exposure

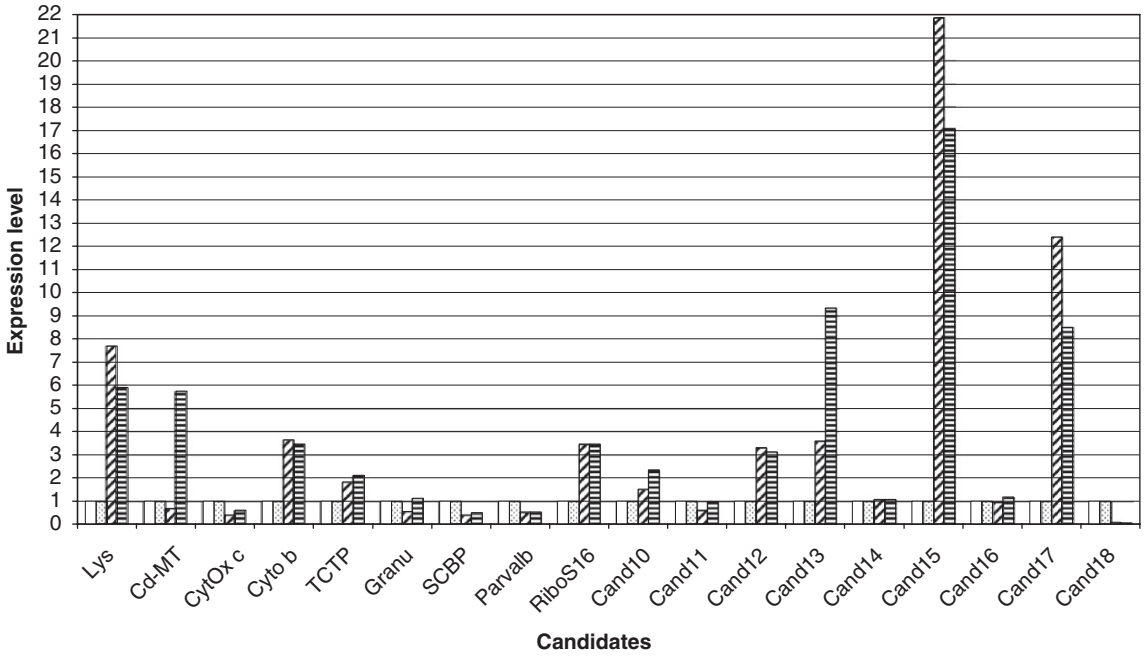

Figure 2 Relative expression ratios of the 18 selected candidates during the metallic exposure. (For each transcript, expression levels (relative to β-actin) were measured on *E. fetida* exposed 1 and 14 days to the metallic mixture (*E*) and on *E. fetida* exposed 1 and 14 days to a control soil (*C*). For better clarity, relative expression is presented as a ratio of expression levels of exposed *E. fetida* relative to unexposed *E. fetida*. □: Control 1 day; ⊡: Control 14 days; ▨: Exposed 1 day; ▤: Exposed 14 days.

(1.52 fold; $p = 0.020$ and 3.93; $p = 0.006$, respectively) compared with the quantity obtained in control animals.

Metal analysis

Cd and Pb burdens of *E. fetida*, which were metal-exposed for 1 or 14 days, indicated clearly that these metals were accumulated in worms exposed either to artificial metal-spiked soil or to polluted field soil. On the other hand, accumulation of Zn in worms was not observed. Indeed, concentrations of Zn kept unchanged in worms exposed to 740 mg kg^{-1} of Zn in metal-spiked soil or 1191 mg kg^{-1} of Zn in polluted field soil (Tables 5 and 6).

Rapid amplification of cDNA ends (RACE)

A 5′RACE was performed on transcripts corresponding to candidate 17 to obtain the full-length sequence. This 5′RACE treatment allowed the obtention of a 1473 bp sequence (initially only 399 bp) containing the complete coding sequence (Genbank accession number EU296921) with strong similarity to the coactosin-like protein (CLP) of *Danio rerio* (*E*-value = 6e−32) (Supplementary Figure 1) (Fig. 4).

Discussion

The Annelida Oligochaeta *E. fetida* was chosen to perform this work because this model is recommended in ecotoxicology by OECD [38] and has been the subject of many physiological studies [17,25,31]. Although studied a lot, the number of nucleotidic sequences available in genomic libraries was relatively low until recently. A total of 3292 sequences are currently available in Genbank, and most of them are due to a single study by Pirooznia et al. [39]. The 764 sequences we obtained add to this initial database and are particularly interesting since they have been specifically chosen so as to be differentially expressed following metallic stress in coelomocytes.

In this work, we focused on the identification of genes whose expression was up-regulated after 1 day as well as after 14 days of exposure and on genes, which were down-regulated after 14 days of exposure. The approach of identifying genes potentially under-expressed after 1 day of exposure was not exploited because a previous study showed that for many genes the decrease of level of expression following metal exposure was only transient. Thus, the level of expression of many genes coding effectors such as catalase, superoxide dismutase, heat shock proteins decreases after 48 h intoxication. This drop of expression was attributed to the massive induction of the gene coding MT [16].

The first validation step consisted of the analysis of the expression level of each candidate using real-time PCR in worms exposed to contaminated artificial soil. This step demonstrated that 9 candidates among the 18 selected were really induced by the contamination. Among the sequences exhibiting a strong redundancy and validated by this first step, it is not surprising to find the Cd–MT. Indeed, this gene, which is well documented in lumbricids, is involved in homeostasis of essential MTE (Cu and Zn) and in detoxification mechanisms of non-essential metals (Cd). 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 metallic contamination and especially to a

Table 3 Induction factors (compared with control soils) and *p* values observed for the 18 selected candidates during the metallic exposure.

		Lys	Cd-MT	CytOx c	Cyto b	TCTP	Granu	SCBP	Parvalb	RiboS16	Cand10	Cand11	Cand12	Cand13	Cand14	Cand15	Cand16	Cand17	Cand18
1 day		7.67	0.68	0.38	3.63	1.81	0.55	0.39	0.51	3.46	1.50	0.59	3.30	3.59	1.05	7.67	0.93	12.38	0.08
		3.10^{-4}	n.s.	0.005	0.006	0.031	0.008	0.010	0.002	0.005	n.s.	0.021	0.007	0.007	n.s.	3.10^{-4}	n.s.	0.003	0.036
14 days		5.89	5.75	0.60	3.45	2.11	1.11	0.50	0.51	3.44	2.34	0.97	3.12	9.32	1.07	5.89	1.18	8.51	0.04
		9.10^{-4}	0.001	0.007	6.10^{-4}	6.10^{-4}	n.s.	0.002	0.001	0.033	0.005	n.s.	0.003	0.029	n.s.	9.10^{-4}	n.s.	0.003	0.023

n.s: non-significant.

contamination containing Cd [15,16,20,40]. In our study, this transcript is used as a positive control and its presence among the potentially induced sequences was expected and is indeed reassuring. The presence of many copies of this transcript also shows that the subtractive step of the SSH was successful, which is necessary to obtain satisfactory results, was correctly carried out and that the 764 single sequences have a good chance to be differentially expressed under the conditions of realization of the subtractive library.

The translationally controlled tumor protein (TCTP) was identified in another Annelida Oligochaeta species (*L. rubellus*) and its expression is induced by a multimetallic contamination [34]. Expression of TCTP in *E. fetida* is not affected by a monometallic exposure to Cd or Cu [16]. Therefore, the present work shows that the expression of TCTP is slightly induced in *E. fetida* exposed to artificial soil containing $40\,mg\,kg^{-1}$ of Cd, $500\,mg\,kg^{-1}$ of Pb and $700\,mg\,kg^{-1}$ of Zn. TCTP was first identified as a tumor-associated protein. It was demonstrated in vertebrates that TCTP is a housekeeping gene and that gene expression deregulation may be involved in tumor development of colon carcinoma [41]. Moreover, it was shown that TCTP displays more general ''cytokine-like activities'', as it also induces the production of interleukins from basophils and eosinophils [42].

The three other candidates presenting homologies with known proteins and showing an increase of their expression level following a metallic stress on artificial soil are lysenin, cytochrome b and ribosomal protein S16. From our knowledge, these effectors have not been involved in a context of regulation of metals or metal pollution. Lastly, among the 9 candidates validated by this first step, candidates 10, 12, 15 and 17 correspond to non-identified genes.

One of the tested candidates, which did not show variation of expression in exposed worms is documented in other species. Indeed, the cytochrome C oxidase subunit I was studied in aquatic organisms. It was demonstrated that cytochrome C oxidase subunit I gene expression was up-regulated after mercury exposure in zebrafish *Danio rerio* [43] and Cd exposure in zebra mussel *Dreissena polymorpha* [44]. Over-expression of cytochrome C oxidase subunit I has also been evidenced in a pyrethroid insecticide-resistant strain of German cockroach *Blatella germanica* [45].

The aim of our study being to consider environmental conditions, a second step of validation of the 9 candidates induced on polluted artificial soil was undertaken. To do this, worms were exposed to a ''naturally'' contaminated soil originating from the site of Metaleurop, north of France.

Some genes that were induced in worms exposed to contaminated artificial soil were not induced in the tested Metaleurop soil. Such differences may be due to the fact that the structure and the texture of artificial soil are completely different compare to field soil (Table 5). For instance, it was shown that the bioavailability of MTE for living organisms and especially worms, depends of soil properties like the content of organic matter, the pH, the cation exchange capacity and the quantity of clay [46,47]. Moreover, the mode of administration of MTE in artificial soil, via the use of metallic solutions, makes these metals much bioavailable. As a consequence, toxicity and a greater bioaccumulation of metals are usually observed for weaker concentrations in artificial soil [48,49].

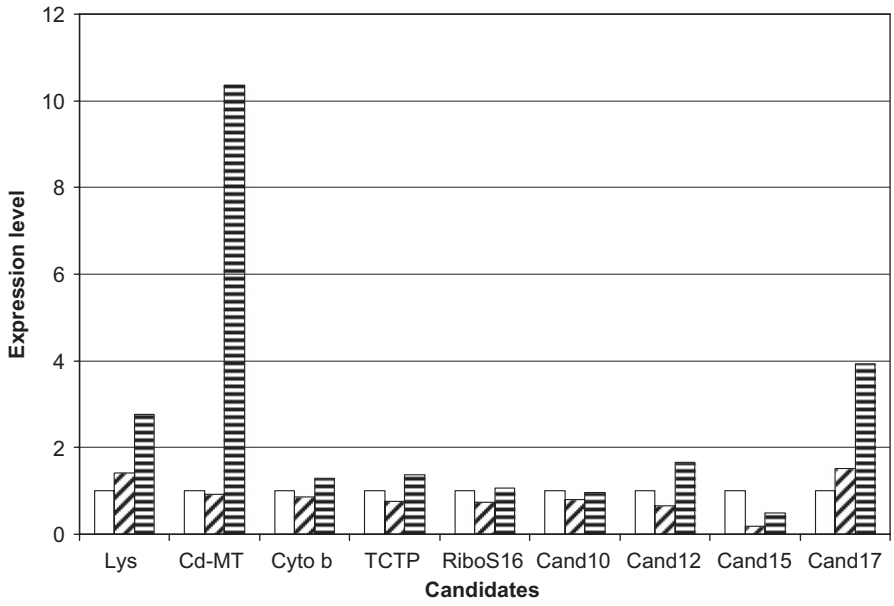

Figure 3 Relative expression ratios of transcripts of 9 remaining candidates during the smelter soil exposure. (For each transcript, expression levels (relative to β-actin) were measured on *E. fetida* exposed 1 and 14 days to the smelter soil (*E*) and on *E. fetida* from controlled cultures of our laboratory (*C*). For better clarity, relative expression is presented as a ratio of expression levels of exposed *E. fetida* relative to unexposed *E. fetida*. □: Control; ▨: Exposed 1 day; ▤: Exposed 14 days.

Table 4 Induction factors (compared with control soils) and *p* values observed for the 18 selected candidates during the smelter soil exposure.

	Lys	Cd-MT	Cyto b	TCTP	RiboS16	Cand10	Cand12	Cand15	Cand17
1 day	1.42	0.93	0.86	0.76	0.73	0.81	0.66	0.18	1.52
p	0.030	n.s.	0.039	n.s.	n.s	0.014	0.013	0.002	0.020
14 days	2.76	10.36	1.29	1.38	1.07	0.96	1.66	0.49	3.93
p	0.012	0.041	0.002	n.s.	n.s	n.s.	0.010	0.006	0.006

n.s: non-significant.

Table 5 0.01 M $CaCl_2$ extractable ($n = 3$) and total ($n = 3$) metal concentrations of soils used in this study.

Soil	0.01 M $CaCl_2$ extraction			Total metal concentrations		
	Cd (mg/kg)	Pb (mg/kg)	Zn (mg/kg)	Cd (mg/kg)	Pb (mg/kg)	Zn (mg/kg)
Metal-spiked soil	9.62	1.24	172.73	41.18	614.7	740.4
Field soil	1.7	1.3	143.8	15.1	946	1191

Cd and Pb burdens of *E. fetida*, which were metal-exposed for 1 or 14 days indicated clearly that these metals were accumulated in worms exposed either to artificial metal-spiked soil or to polluted field soil. These data showed that gene expressions are induced in animals that accumulate metals (cadmium and lead). Zn remains unchanged in worms exposed to metal-spiked soil or in polluted field soil. These results are in accordance with those of Demuynck et al. [27] suggesting that Zn is regulated.

Overall, only 3 candidates were induced in worms exposed to the Metaleurop polluted soil. These candidates are, Cd–MT, lysenin and a transcript corresponding to a gene, which function was unknown (candidate 17).

Cloning of the complete coding sequence of candidate 17 allowed the identification of the CLP. CLP is a member of the ADF/Cofilin group of actin-binding proteins, which support the activity of the 5-Lipoxygenase (5-LO), an enzyme of central importance in cellular leukotriene (LT) synthesis. 5-LO and LT are two key components involved in

Table 6 Metal contents (Cd, Pb and Zn) in non-exposed *Eisenia fetida* and in *Eisenia fetida* exposed either to artificial metal-spiked soil or to polluted field soil.

	Cd (mg/kg of dry worm)	Pb (mg/kg of dry worm)	Zn (mg/kg of dry worm)
Control animals	0.63 ± 0.50	< 0.01	91.04 ± 3.79
Metal-spiked soil 1 day	6.82 ± 1.38	6.75 ± 2.77	175.52 ± 9.01
p	< 0.05	< 0.05	n.s.
Metal-spiked soil 14 days	33.12 ± 2.36	47.83 ± 4.27	184.66 ± 16.61
p	< 0.05	< 0.05	n.s.
Field soil 1day	4.86 ± 0.50	9.05 ± 2.84	156.11 ± 7.18
p	< 0.05	< 0.05	< 0.05
Field soil 14 days	23.22 ± 4.67	32.26 ± 4.50	95.26 ± 12.35
p	< 0.05	< 0.05	n.s.

Results are expressed as mean values ± standard errors ($n = 3$ groups of 5 animals per treatment). n.s: non-significant.

```
Multiple sequence alignment

H_sapiens       MATKIDKEACRAAYNLVRDDGSAVIWVTFKYDGS-TIVPGEQGAEYQHFIQQCTDDVRLF 59
M_musculus      MATKIDKEACRAAYNLVRDDGSAVIWVTFRYDGA-TIVPGDQGADYQHFIQQCTDDVRLF 59
D_rerio         MATRIDKEACREAYNLVRDDSSGICWACFKYDGS-TIVPGGHGSDYEEFKSQCTVDSRVF 59
E_fetida        MAD-IQKESIAEAYDDVRNDQTPTTWALLGYDDSNTIVLLGTGSEYEEFRSKFSDDDRLF 59
                **  *:**:   **: **:* :   *. : **.: ***    *::*:.* .: : * *:*

H_sapiens       AFVRFTTGDAMSKRSKFALITWIGENVSGLQRAKTGTDKTLVKEVVQNFAKEFVISDRKE 119
M_musculus      AFVRFTTGDAMSKRSKFALITWIGEDVSGLQRAKTGTDKTLVKEVVQNFAKEFVISDRKE 119
D_rerio         GFVRIMTGDAMSKRSKFTFITWIGENITGLQRAKISTDKALVKDAVPTFAKEFMISDPKE 119
E_fetida        GFVRLTAGDELSKRAKFVLITWIGSNISALKRAKVSTDKSTVKSILQNFALEIQISDLAE 119
                .***: :** :***:**.:*****.::::.*:*** .***: **. : .** *: ***  *

H_sapiens       LEEDFIKSELKKAGGANYDAQTE- 142
M_musculus      LEEDFIRSELKKAGGANYDAQSE- 142
D_rerio         LEEEYLRTELKKAGGANYDAQAE- 142
E_fetida        LEEPNIREALKKAGGAQYGTGVRD 143
                ***  ::  *******:*.:  .
```

Figure 4 Alignment of the *Eisenia fetida* coactosin-like protein putative amino acid sequence and other selected amino acid sequences using ClustalW: *Homo sapiens* (Accession no. Q14019), *Mus Musculus* (Accession no. NP_082347), *Danio rerio* (Accession no. NP_956306) and *Eisenia fetida* (Accession no. EU296921). Amino acids in bold indicate the two binding sites of coactosin-like protein. *Symbols*: * identity, **:** conserved substitutions have been observed, . semi-conserved substitutions are observed.

inflammatory disorders in vertebrates [50,51]. Studies on immune cells in human and vertebrate models have shown that CLP can up-regulate and modulate the 5-LO pathway *in vitro* [51].

Thus, CLP support Ca^{2+}-induced 5-LO activity and can function as a chaperone and/or scaffold for 5-LO. Moreover, CLP up-regulate the LT production, a very important agent in inflammatory response [51, see 52 for details].

It has been shown in rabbit macrophages, that Cd^{2+} disrupts the 5-LO pathway by enhancing release of LTB_4, responsible for the extension of the inflammatory reaction after cadmium exposure [53]. In the latter study, authors suggest that Cd^{2+} partially mimics the effect of Ca^{2+}, which is an activator of the 5-LO activity and consequently required for the synthesis of LTB_4. An indirect action of cadmium is supported by the lack of metal response elements observed in upstream part of mouse CLP gene [54]. Little is known about CLP in invertebrates. Therefore, the presence of CLP in *E. fetida* immune cells also supports its implication in stress and/or inflammatory mechanisms. Moreover, the conservation of the two residues of key importance for interaction with 5-LO (Lys-131) and F-actin (Lys-75) is in favour of a similar role for CLP in our invertebrate model (Figure 4) [55].

Qualitative and quantitative gene expression analysis may be used to study the immune part of the response to a metallic stress. Moreover, expression profiles of numerous selected genes, as MT and both new ones described in this work, may constitute a ''signature'' of changes due to MTE.

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Appendix A. Supporting Information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.dci.2008.06.009.

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