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Exploring DNA methylation patterns in copper exposed *Folsomia candida* and *Enchytraeus crypticus*

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ABSTRACT

Accumulating evidence shows that epigenetics-mediated phenotypic plasticity plays a role in an organism's ability to deal with environmental stress. However, to date, the role of epigenetic modifications in response to stress is hardly investigated in soil invertebrates. The main objective of this proof of principle study was to explore whether total cytosine and locus-specific CpG methylation are present in two important ecotoxicological model organisms, the springtail *Folsomia candida* and the potworm *Enchytraeus crypticus*, and if so, whether methylation patterns might change with increased toxicant exposure. LC–MS/MS analyses and bisulfite sequencing were performed to identify the CpG methylation state of the organisms. We show here, for the first time, a total level of 1.4% 5-methyl cytosine methylation in the genome of *E. crypticus*, and an absence of both total cytosine and locus-specific CpG methylation in *F. candida*. In *E. crypticus*, methylation of CpG sites was observed in the coding sequence (CDS) of the housekeeping gene Elongation Factor 1α, while the CDS of the stress inducible Heat Shock Protein 70 gene almost lacked methylation. This confirms previous observations that DNA methylation differs between housekeeping and stress-inducible genes in invertebrates. DNA methylation patterns in *E. crypticus* were not affected by exposure to copper (II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$) mixed in with LUFA 2.2 soil at sublethal effect concentrations that decreased reproduction by 10%, 20% and 50%. Although, differences in CpG methylation patterns between specific loci suggest a functional role for DNA methylation in *E. crypticus*, genome-wide bisulfite sequencing is needed to verify whether environmental stress affects this epigenetic hallmark.

1. Introduction

Many studies have shown that natural populations contain a significant amount of genetic variation upon which environmental stress factors may act (Roelofs et al., 2008). For instance, a study on the springtail *Orchesella cincta* showed distinct stress-induced gene expression in animals not acclimated to metals. In contrast, significantly elevated constitutive as well as cadmium-induced metallothionein expression was found in animals that gained tolerance to metals, suggesting adaptation through increased constitutive cadmium detoxification (Roelofs et al., 2008). For a long time scientists have been focusing on genetic adaptation due to changes in nucleotide sequence of protein-encoding genes or their promoter regions. However, recent studies indicate that environmental stress can influence not only DNA sequences as such, but also the epigenetic markers associated with the DNA (Kille et al., 2013; Pierron et al., 2014). Epigenetics is the study of changes in the expression and function of genes that cannot be attributed to changes in DNA sequence (Richards et al., 2010; Head et al., 2012). Epigenetic markers that are associated with these changes include DNA methylation, histone modifications, nucleosome remodeling, and non-coding RNA-mediated transcriptional regulation (Fuks, 2005; Goldberg et al., 2007; Bossdorf et al., 2008). The most intensely investigated marker is DNA methylation, which consists of an addition of a methyl group (-CH3) to the fifth position at the cytosine nucleotide in DNA (Vandegehuchte and Janssen, 2011; Head, 2014).

DNA methylation is associated with a number of key processes such as development, genomic imprinting and cell differentiation (Regev et al., 1998; Marhold et al., 2004; Vandegehuchte et al., 2009a). A number of studies have provided convincing evidence that changes in DNA methylation patterns are associated with environmental stress and

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may result in physiological alterations. For example, exposure to soluble fractions of industrial waste resulted in significant DNA hypermethylation and induced oxidative damage in Nile tilapia (*Oreochromis niloticus*). Furthermore, increased rates of malondialdehyde (indicates the process of lipid peroxidation) in *O. niloticus* erythrocytes suggested a disturbance in lipid membranes in response to the contamination (Flohr et al., 2012). Another study showed that a contaminated environment contributed to a decrease in total cytosine methylation in the flatfish *Limanda limanda* and enabled the development of hepatocellular adenoma tumors (Mirbahai et al., 2011).

Adverse effects associated with altered methylation status have also been observed in invertebrates. For instance, genome-wide methylation analysis of *Daphnia magna* exposed to the toxic cyanobacterium *Microcystis aeruginosa* revealed a complex mechanistic response with differential methylation patterns enriched for serine/threonine amino acid codons and genes related to protein synthesis and transport (Asselman et al., 2017). Serine/threonine amino acids play an important role in stress response as they regulate protein folding. In response to *Microcystis* stress, differentially methylated genes corresponded with genes that are likely to be alternatively spliced. The authors suggested that DNA methylation plays an important role in the animal's response to toxicity as it is regulated by environmental stress (Asselman et al., 2017).

To date, the role of epigenetic modifications in response to stress is hardly investigated in soil dwelling organisms. Kille et al. (2013) reported epigenetic effects of environmental contamination (i.e. arsenic) on the earthworm *Lumbricus rubellus*. Methylation sensitive AFLP (Me-AFLP) analysis revealed a distinction between two lineages within the sampled earthworms. Lineage A did not show any association with soil arsenic concentrations. However, DNA methylation patterns were correlated with soil arsenic levels in lineage B, suggesting a function for DNA methylation in the adaptation to environmental contamination at least in some lineages.

In this proof of principle study, we aimed to explore whether total cytosine and locus-specific CpG methylation can be detected under normal homeostasis in the soil dwelling organisms *Folsomia candida* and *Enchytraeus crypticus*, and moreover, if methylation patterns might change with increased toxicant exposure. In more detail, we assessed whether CpG methylation patterns could be associated with the stress response to copper exposure, a metal that has previously been reported to cause toxic effects in springtails (Pedersen and Van Gestel, 2001) and enchytraeids (Gomes et al., 2012). Total cytosine methylation was measured as the ratio between deoxy guanosines and deoxy 5 methylated cytosines after total digestion of genomic DNA using liquid chromatography tandem mass spectrometry (LC–MS/MS). Additionally, CpG methylation of specific loci of gene bodies in the housekeeping gene elongation factor 1 alpha (Ef1α) and the stress-inducible gene heat-shock protein 70 (Hsp70) were investigated using bisulfite sequencing, as CpG methylation in invertebrates is mainly found in gene bodies (exons and introns), where housekeeping genes show in general elevated methylation levels associated with high constitutive expression (Lyko et al., 2010; Wang et al., 2013). Such data will indicate whether methylation could potentially vary between a housekeeping gene and a stress-response gene in our models. On the basis of these studies in invertebrates we hypothesize that housekeeping genes with high constitutive expression show high gene body methylation, while inducible stress response genes show lower levels of gene body methylation. Finally, we attempted to assess the influence of copper toxicity on the observed methylation patterns.

2. Materials and methods

2.1. Test compound and spiking of soil

Copper (II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$) (99% purity) was obtained from Merck (Darmstadt, Germany). LUFA 2.2 soil (Speyer, Germany) was used as a control/reference. This soil has a reported total organic carbon content of 2.09%, pH_{CaCl2} of 5.5 and a water holding capacity (WHC) of 46.5%. To obtain contaminated soils, LUFA 2.2 was spiked with dry powder using three effect concentrations (ECx) that affected reproduction by 10 (EC10), 20 (EC20) and 50 (EC50) percent compared to the untreated control. For *E. crypticus*, EC10, EC20 and EC50 values for the effect of $CuCl_2$ (literature data on $CuSO_4$ exposure in *E. crypticus* is absent) of 111, 159 and 293 mg Cu/kg dry soil were established from a range finding test (0–35–70–120–300 mg Cu/kg dry soil) performed by Menezes-Oliveira et al. (2011). Literature on $CuSO_4$ toxicity to *F. candida* showed an EC50 of 519 (CI: 21–13095) mg Cu/kg dry soil (Bruus Pedersen et al., 2000). To narrow down the range of the confidence intervals we performed a toxicity test with *F. candida* ourselves; see below. This test provided EC10, EC20 and EC50 values of 188, 319 and 782 mg Cu/kg, respectively. These values were used for further analyses. All soil samples were moistened with deionized water to reach a moisture content equivalent with 50% of the WHC.

2.2. Toxicity tests

The parthenogenetic springtail *F. candida* ("Denmark strain", VU Amsterdam) and the annelid worm *E. crypticus* (VU Amsterdam) were used as model organisms. The cultures of both species were kept in a climate room at 16 ± 0.5 °C and a 16:8 h light:dark regime.

A 28-day toxicity test with *F. candida* was performed following OECD guideline 232 (OECD, 2009), using 100 ml glass jars. Springtails were exposed to $CuSO_4{\cdot}5H_2O$ at concentrations of 0–100–200–400–800–1600 mg Cu/kg dry LUFA 2.2 soil in order to determine EC10, EC20 and EC50. For each treatment (control and all concentrations) five replicates were prepared. Springtail juveniles of synchronized age (10–12 days old) were used for the experiment. After two days of soil equilibration, ten animals were introduced into each test jar, which was closed with a plastic screw top. Once a week jars were aerated, soil was moistened with deionized water and animals were fed dried baker's yeast. Springtails were harvested by adding 100 ml of deionized water to each test jar, gently stirring and transferring them to a plastic beaker, allowing springtails to float on the surface. Pictures were taken of the surface, used later to count all animals with the software program ImageJ (version 1.49) so as to determine reproduction (number of juveniles). EC10, EC20 and EC50 values were determined by using a logistic dose response model; corresponding 95% confidence intervals were calculated by using non-linear regression analysis in IBM SPSS Statistics 23 software (Corp, 2015). Subsequently, a second toxicity test was performed similar to the previous test using the same number of replicates, where springtails were exposed to concentrations corresponding to these effect levels. After 28 days, juveniles produced from this second test were collected, snap frozen and stored at −80 °C for later epigenetic analyses.

The test with *E. crypticus* followed OECD guideline 220 (OECD, 2004). Animals were exposed to previously obtained effect concentrations (EC10, EC20 and EC50) (Menezes-Oliveira et al., 2011). Five replicates were prepared for each treatment (control, EC10, EC20 and EC50). Enchytraeids were selected based on their size (0.4–0.6 cm) and visible clitellum (indicating adulthood). Ten animals were introduced into each 100 ml glass jar containing 30 g of moist soil. Jars were closed with perforated aluminum foil and once a week all jars were aerated, soil moistened with deionized water and animals were fed with 2 mg oatmeal. After 3 weeks, tap water was added to jars containing enchytraeids, after which the animals could be collected from the water using a small hook. Collected enchytraeid juveniles were snap frozen and stored at −80 °C for later DNA analyses.

All toxicity tests were performed in climate room at 20 ± 0.5 °C, 75% relative humidity and a 16:8 h light:dark regime.