Effect of cadmium on bacterial communities in the gut of *Pontoscolex corethrurus*

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ABSTRACT

Endogeic earthworms are often exposed to various kinds of stress which in turn could have a profound effect on the micro flora of its gut. In this study, we investigated the effect of cadmium stress on the bacterial communities of the gut of endogeic earthworms *Pontoscolex corethrurus*. Two bacterial species *Citrobacter intermedius* and *Neisseria mucosa* were selectively accumulated in the gut of earthworms that were exposed to cadmium stress when compared to the control earthworms. When the microorganisms were further studied for their tolerance for cadmium *in-vitro,* these microorganisms were able to tolerate the metal even at higher concentrations. This study highlights the importance of association between earthworms and microorganisms with respect to bioremediation of soils contaminated with heavy metals.

Keywords: *Pontoscolex corethrurus*, endogeic, heavy metals, bioaccumulate, bioremediation

INTRODUCTION

The heavy-metal cations play an important role as trace elements in biochemical reactions. At higher concentrations, however, heavy-metal ions form unspecific complex compounds in the cell, which lead to toxic effects. Heavy metals released into the environment tend to persist, accumulating throughout the food chain, thus posing a serious threat to the environment, animals and humans. Thus, heavy metal resistance is a general demand of every living cell.

Cadmium is one of the largest three heavy metal poisons and has no known essential biological function. In its compounds, Cd occurs as the divalent Cd (II) ion. Cadmium in the body is known to affect several enzymes [1]. High concentrations of Cd in rice grain and tobacco leaves is of particular concern for humans [2]. This heavy metal is primarily accumulated in the kidney in human body which causes renal toxicity; renal dysfunction and bone impairment [3]. Food intake and tobacco smoking are the main routes by which Cd enters the body [4].

Assessing pollutants in different components of the ecosystem has become an important task in preventing risk to natural life and public health. Bio-monitoring schemes using indicator species to estimate the levels of contaminants in different parts of the ecosystem have been developed. The earthworms have been proved to be sensitive indicators of contaminated soils. They are eco-system engineers and have been involved in bioremediation for many years. Endogeic earthworms like *Pontoscolex corethrurus* are a major component of soil faunal communities in ecosystems of the tropics [5]. They bio-accumulate the heavy metals, detoxify them and improve the quality of soil [6-9]. The heavy metals are taken up by the earthworms either by immobilization in the cells of gut wall or by storing in waste nodules formed within body cavity or by excretion through calciferous glands [10].

Due to the selective pressure from the metal in the growth environment, microorganisms have evolved various mechanisms to resist the heavy metal stress. Microorganisms are known to tolerate and accumulate a wide range of heavy metals which are the common pollutants of soil. They are ideally used in bioremediation because they possess enzymes that allow them to use environmental contaminants as food. The earthworms harbor millions of microbes in their gut. The gut micro flora of earthworms have been known to perform important roles like de-nitrification (in nitrogen fixation) and production of degradative enzymes (for decomposition of organic matter) [11].

This study investigates the interaction between the endogeic earthworms *Pontoscolex corethrurus* and their gut microflora in uptake of heavy metals from the polluted soil samples. In this study, we investigated the effect of cadmium stress on the microbial communities of the gut of endogeic earthworms (*Pontoscolex corethrurus)*. The endogeic earthworms have been selected for the study because they live and feed in the mineral soil layers. Hence they are in constant contact with the polluted soil and are directly influenced by the presence of metal pollutants in the soil which in turn could have a profound effect on the micro flora of their gut.

MATERIALS AND METHODS

Collection of Earthworms

Earthworms (*Pontoscolex corethrurus*) were collected from the garden soil during the monsoons and maintained in big aerated containers (32x29x27cm) under normal conditions for a period of two weeks. Humus was prepared using soil and dried leaves for maintaining the earthworms in the laboratory under optimal conditions needed for their survival. To maintain uniform initial conditions in the gut, the gut of the earthworms was cleared by feeding them on wet filter paper for a period of 48 hours inside a box.

Exposure of earthworms to heavy metal - cadmium

Boxes (21x18x9cm) were filled with one kg of soil. Six earthworms of uniform size were weighed and introduced to increasing concentrations of cadmium (0, 40, 80,160, 320 and 640 mg/kg dry wt. of soil) used as chloride salt ($CdCl_2$) for a period of 15 days. All the concentrations were maintained in triplicates. The soil without any addition of metal was used as control. The soil was covered with a thin layer of humus. The boxes were punched for adequate aeration. The earthworms were maintained with adequate moisture content (40%) and were regularly monitored for % weight gain/loss and mortality on days 3, 8, 10, and 15.

Preparation of gut homogenate

The earthworms surviving the cadmium treatment were used for preparation of gut homogenate to isolate the gut micro flora. They were surface sterilized by swabbing gently with 70% alcohol. The region below the gizzard (Intestine) was divided into three parts as anterior, middle and posterior parts of the intestine. Each gut portion was separately homogenized in two ml of sterile distilled water and centrifuged to obtain the supernatant. The supernatant was stored at 4°C and was used for further microbiological analyses. The gut homogenate of control earthworms was prepared similarly.

Characterization of bacteria from the gut homogenate

The homogenate obtained was diluted 10-folds and 0.1 ml of diluted sample was spread plated on the nutrient agar plates. The plates were incubated in an inverted position at 37°C for 24h. Based on the physical characteristics the colonies which were morphologically distinct were further streaked to obtain pure cultures. The pure cultures were characterized based on their physical characteristics and using different biochemical assays like Gram Staining, IMViC Test, Catalase test, Oxidase test, Urease test, Nitrate reductase test and Sugar fermentation assays for the identification of the species [12].The microbes isolated from the gut of earthworms subjected to metal treatment were compared with the gut micro flora of control earthworms to identify the bacteria specifically harbored in the gut of earthworms subjected to cadmium treatment.

Study of tolerance of selectively accumulated gut bacteria to cadmium stress

After the comparison and analysis of the differences in the gut micro flora of the earthworms, *Citrobacter intermedius* and *Neisseria mucosa* were found to be selectively accumulated in the gut of earthworms exposed to cadmium stress. The tolerance of these microorganisms to cadmium was analyzed *in vitro* by studying the growth patterns of these organisms under the heavy metal stress (Cd: 0, 0.5, 1, 2, 4,8mg/L of medium). A loop of pure culture (of each of these micro organisms) was inoculated into 50ml of the nutrient broth and kept on a shaker for 16h at 37°C. This culture (0.5%) was then used as the seed culture to inoculate the series of culture flasks. The culture flasks were maintained on a shaker at specified temperature. Bacterial growth was measured as increase in turbidity of the culture by measuring optical density at 600nm using a spectrophotometer. Absorbance values of the culture were taken every one hour until stationary phase was attained. The obtained absorbance values were plotted on the graph against time to obtain the growth curve.

RESULTS AND DISCUSSION

Effect of exposure of earthworms to cadmium

Cadmium had a deleterious effect on growth and development of the earthworms. All the eathworms that were introduced in the soil spiked with Cadmium salt, irrespective of its concentration, showed decrease in their weights by day3. The earthworms exposed to concentration of Cd more than 80mg/kg were found dead within 8 days (Table 1). It is calculated as given below:

$$\left\{ \frac{\text{Avg. wt. on the respective day} - \text{avg. wt. on day 0}}{\text{Avg. wt. on day 0}} \right\} * 100$$

Isolation and characterization of the gut bacteria

A dilution of the homogenate obtained from the earthworms was used for the isolation of the gut microbes. Upon spread plating, various colonies were observed as shown (Figure-1). These colonies were physically characterized on the basis of various morphological features and were streaked to obtain pure culture and subjected to Gram's staining (Table 2). Depending upon the Gram's nature and morphology of the micro organisms, they were subjected to a series of different biochemical tests. The results of the biochemical characterization of the bacterial colonies are displayed (Tables 3 and 4). Based on the biochemical characterization of the isolated bacterial cultures they were identified (Table 5).

Table 1. Effect of cadmium on the earthworms *Pontoscolex corethrurus.*

Concentration of salt $CdCl_2$ (mg/kg of dry wt. of soil)	Average weight & percentage weight gain/ loss on the days									
	0th		3rd		8th		10th		15th	
	g	%	g	%	g	%	g	%	g	%
0	0.19 (18)	100	0.19 (17)	0	0.23 (17)	+21.1	0.22 (16)	+15.8	0.2 (14)	+5.26
40	0.16 (18)	100	0.14 (18)	-12.5	0.14 (15)	-12.5	0.15 (12)	-6.25	0.17 (11)	+6.25
80	0.14 (18)	100	0.12 (18)	-14.3	0.11 (12)	-21.4	0.12 (11)	-14.3	0.13 (05)	-7.14
160	0.13 (18)	100	0.1 (15)	-23.1	0	0	0	0	0	0
320	0.13 (18)	100	0.09 (12)	-30.8	0	0	0	0	0	0
640	0.15 (18)	100	0.08 (11)	-46.7	0	0	0	0	0	0

a: Average weight of the earthworms alive on the day of observation. The number in parentheses indicates the number of the earthworms alive on the day of observation.
b: Percentage change in the average weight of the earthworms compared to the 0th day.

Table 2. Gram's staining results of the bacteria isolated from gut of earthworms exposed to Cadmium.

Colony code[a]	Gram's nature[b]	Morphology of cells
CCdFI	GN	Bacillus
CCdFII	GN	Bacillus
CCdM	GN	Bacillus
CCdH	GN	Bacillus
Cd1FI	GN	Bacillus
Cd1FII	GN	Bacillus
Cd1FIII	GN	Coccus
Cd1MI	GN	Bacillus
Cd1MII	GN	Coccus
Cd1H	GN	Bacillus
Cd2F	GN	Bacillus
Cd2MI	GN	Bacillus
Cd2MII	GN	Coccus
Cd2MIII	GN	Bacillus
Cd2H	GN	Bacillus

a: Colony ID : F-Foregut; M-Midgut; H-Hindgut; b:GP: Gram positive; GN: Gram negative

As evident from the Table 5, it is seen that a few of the microorganisms were retained only in specific regions of the gut. Bacterial species which were identified under metal stress were compared with those in control to identify the bacteria that were enriched or selectively accumulated under metal stress. This comparison led to the identification of two bacterial species for Cadmium stress- *Citrobacter intermedius* and *Neisseria mucosa*. These bacteria were further checked for their efficiency of metal tolerance.

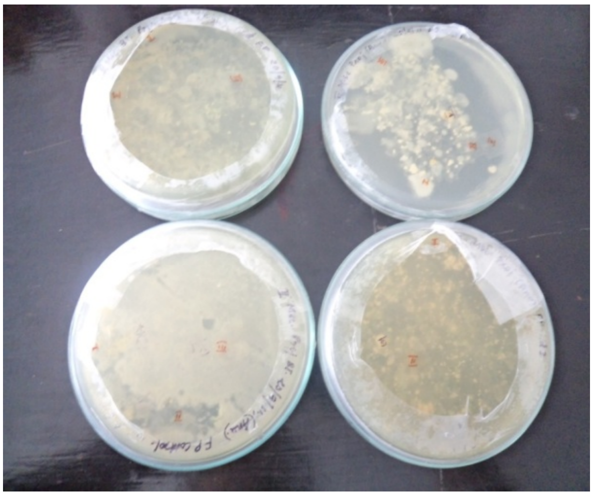

Figure 1. Colonies obtained after spread plating of the gut homogenate obtained from earthworms that survived the copper metal stress.

Table 3. Biochemical tests performed for Gram negative bacilli isolated from gut homogenate of earthworms under Cd stress.

Colony code	Lactose	Glucose	Indole	Citrate	Mr	H2s	Urea	Organism
CCdFI	P	NA	P	N	NA	NA	NA	*Escherichia coli*
CCdFII	P	NA	N	NA	P	NA	N	*Klebsiella ozaenae*
CCdM	N	P	N	NA	NA	NA	N	*Proteus inconstans*
CCdH	N	P	P	NA	NA	N	NA	*Proteus rettgeri*
Cd1FI	P	NA	P	P	NA	NA	NA	*Citrobacter intermedius*
Cd1FII	P	NA	P	N	NA	NA	NA	*Escherichia coli*
Cd1MI	P	NA	P	P	NA	NA	NA	*Citrobacter intermedius*
Cd1H	P	NA	P	P	NA	NA	NA	*Citrobacter intermedius*
Cd2F	P	NA	P	P	NA	NA	NA	*Citrobacter intermedius*
Cd2MI	P	NA	P	P	NA	NA	NA	*Citrobacter intermedius*
Cd2MIII	P	NA	N	NA	P	NA	N	*Klebsiella ozaenae*
Cd2H	P	NA	P	P	NA	NA	NA	*Citrobacter intermedius*

NA; Not Applicable for identification, N- negative; P- positive for the test.

Table 4. Biochemical tests performed for Gram negative Cocci isolated from gut homogenate of earthworms under Cd stress.

Colony code	Glucose	Nitrate reduction	Organism
Cd1FIII	P	P	*Neisseria mucosa*
Cd1MII	P	P	*Neisseria mucosa*
Cd2MII	P	P	*Neisseria mucosa*

P- Positive for the test

Table 5. Bacteria identified in the gut of the earthworms under Cadmium stress.

Colony ID	Organism
CcdFI	*E. coli*
CcdFII	*K. ozaenae*
CcdM	*P. inconstans*
CcdH	*P. rettgeri*
Cd1FI	*C. intermedius**
Cd1FII	*E. coli*
Cd1FIII	*N. mucosa**
Cd1MI	*C. intermedius**
Cd1MII	*N. mucosa**
Cd1H	*C. intermedius**
Cd2F	*C. intermedius**
Cd2MI	*C. intermedius**
Cd2MII	*N. mucosa**
Cd2MIII	*K. ozaenae*
Cd2H	*C. intermedius**

*: the organisms that were selectively accumulated under metal stress

Study of tolerance of selectively accumulated gut bacteria to cadmium

The bacteria *C. intermedius* and *N. mucosa* were grown under Cd stress (Table 6) and observed for any significant changes in the growth pattern as a result of the Cu stress (Figure 2). As presented in Figure 2, *C. intermedius*, reached stationary phase after sixteen hours in all the different concentrations of Cd, just the same as control. Even the absorbance values were found to be similar to those of the control. This indicates that *C. intermedius* is not being affected by the increasing concentrations of cadmium and is very tolerant to the heavy metal. The growth curve of *N. mucosa* showed some difference in the absorbance values between the control and the metal-exposed culture broth. The stationary phase was attained at 16-18 hours. (Sixteen for control, eighteen for highest Cd concentration) and there was a decrease in growth of the bacteria with increase in the concentration of cadmium. There was a decrease of almost 15 % growth towards the end of log phase for the control condition and *N. mucosa* grown in 8mg/l cadmium containing media.

Upon exposure to the heavy metals, the earthworms have shown enrichment of a few bacteria by harboring them in their gut in higher amounts than in control. The presence of these selective bacteria or enrichment of bacteria under stress conditions could imply their positive role in aiding the earthworm's tolerance to the metal. This also indicates an association between the earthworms and the microorganisms to bio-remediate the toxic conditions created by heavy metals like cadmium. These differentially accumulated organisms may be present in the soil but there might not have been any interaction between the earthworm and these bacteria. Upon exposure to the metal, the organisms might have been taken up and retained by the earthworms that ultimately conferred them the resistance to the metal. Several bacteria are known for their tolerance to metals which is a result of various metabolism-dependant and –independent processes enabling the metal accumulation.

Citrobacter spp. has been observed to accumulate many heavy metals like copper, cadmium [13] lanthanum, uranyl ion [14] mercury, silver, zinc, and lead [15]. *Citrobacter*, when exposed to growth inhibitory concentration of the heavy metals, overproduces the enzyme, phosphatase, which plays an important role in the accumulation of heavy metals. The acid type phosphatase mediates accumulation of the heavy metals in the form of its phosphates. The enzyme liberates HPO_4^{2-} from glycerol-2-phosphate and the Cd^{2+} ion combines with HPO_4^{2-} to form insoluble $CdHPO_4$, thus leading to the precipitation of the metal and thus metal detoxification[16]. *Neisseria mucosa* has been reported to have the potential to reduce arsenate and selenate oxyanions [17].However no studies reporting its efficiency to tolerate cadmium have been reported as yet.

Table 6. Different concentrations of the Cadmium salt used to study the tolerance of selected bacteria.

Flask ID	Conc. of Cadmium salt (mg/L)
Blank	NA
Control	0
A	0.5
B	1.0
C	2.0
D	4.0
E	8.0

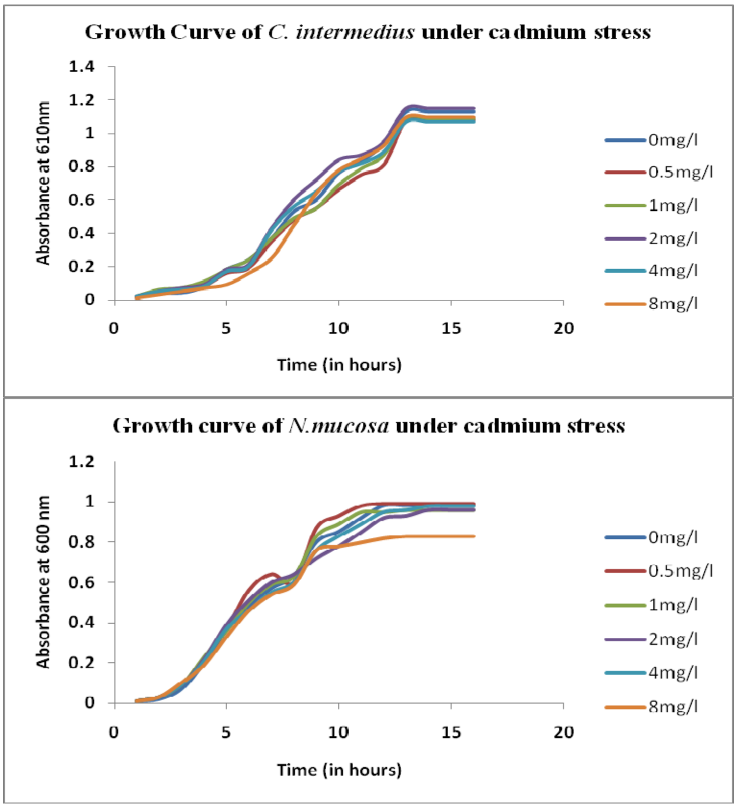

Figure 2. Tolerance of selectively accumulated gut bacteria to cadmium.

Presence of binding proteins like Metallothioneins (MTs) and Phytochelatins(PCs) may contribute for metal uptake which are under the control of genes that may get triggered on exposure to heavy metals [18]. Apart from these chromosomal gene products, there are several microorganisms that perform detoxification with the aid of genes present on the plasmids. The observed changes in the bacterial communities of the earthworms could be used as an indication for the possible contamination of the soil areas, and the differentially accumulated bacteria could be used as bio-remediators to clean up the contaminated sites. However, further research on the extent of tolerance and mechanism of the tolerance of heavy metals by these bacteria has to be done before their probable use in bioremediation. This will help in the understanding of the organism's capability of bioremediation and also throws light on the various methods that can be adapted to enhance the efficiency.

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