Research Journal of Recent Sciences ISSN 2277-2502
Vol. 3(**ISC-2013**), 380-384 (**2014**) *Res. J. Recent. Sci.*

Effect of zinc on Bacterial communities in the Gut of *Pontoscolex corethrurus*

Mallika Mahesh[1], Anu Sharma[1] and Syed ShamimulHasan[2]
[1]Department Of Biotechnology, Mount Carmel College, 58, Palace Road, Vasanth Nagar, Bangalore, INDIA
[2]Life Sciences, School Of Sciences, IGNOU, Maidan Garhi, New Delhi, INDIA

Available online at: www.isca.in, www.isca.me
Received 30th November 2013, revised 16th January 2014, accepted 18th March 2014

Abstract

Microorganisms are known to tolerate and accumulate a wide range of heavy metals which are the common pollutants of soil. The earthworms, being the significant inhabitants of soil, bio-accumulate the heavy metals, detoxify them and improve the quality of soil. The gut micro flora of earthworms has been known to perform important roles like de-nitrification (in nitrogen fixation) and production of degradative enzymes (for decomposition of organic matter). 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. Zinc is one of the largest heavy metal pollutants of the soil. In this study, we investigated the effect of zinc stress on the microbial communities of the gut of endogeic earthworms –Pontoscolex corethrurus. The earthworms were subjected to zinc stress at increasing concentrations for a period of 15 days. Zinc had a deleterious effect on growth and development of the earthworms. It was observed that two bacterial species Aeromonas hydrophila and Bacillus cereus were selectively accumulated in the gut of earthworms that were exposed to metal stress when compared to the control earthworms. This study highlights the importance of association between earthworm and microorganisms with respect to bioremediation of soils contaminated with heavy metals.

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

Introduction

Heavy-metals have an important role as trace elements in many biochemical reactions. At higher concentrations heavy-metal ions form complex toxic compounds in the cell. The heavy metals released into the environment accumulate throughout the food chain and pose a serious threat to the environment, animals and humans. Thus, heavy metal resistance is a general demand of every living cell.

Zinc is one of the largest heavy metal pollutants of the soil. The free zinc ion in water is highly toxic to plants and animals like fish. When zinc enters the bodies of these fish; it leads to bio magnification through the food chain[1]. However, zinc is important in forming complexes such as zinc fingers in DNA and as a component in cellular enzymes[2]. Zinc is used in different industries for plating of iron and alloys such as brass. Zinc carbonate and zinc gluconate are used as dietary supplements and zinc chloride is used in deodorants. In humans, prolonged exposure of heavy metals such as cadmium, copper, lead, nickel, and zinc causes deleterious health effects[3]. Very high levels of zinc can affect the pancreas and the protein metabolism. Extensive exposure to zinc chloride can cause respiratory disorders[4].

Assessing pollutants in different components of the ecosystem can prevent 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 [6789]. 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].

Microorganisms have evolved various mechanisms to resist the heavy metal stress because of the selective pressure from the metals in the growth environment. 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 have the 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. The effect of zinc stress on the microbial communities of the gut of endogeic earthworms was studied. 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.

Material 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-zinc: Boxes (21x18x9cm) were filled with one kg of soil. Six earthworms of uniform size were weighed and introduced to increasing concentrations of zinc (0, 400, 800, 1600, 3200 and 6400 and 13200 mg/kg dry wt. of soil) used as chloride salt ($ZnCl_2$) 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 days0, 3, 6, 10, and 15.

Preparation of gut homogenate: The earthworms surviving the zinc 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 zinc treatment.

Ribotyping of the selected microorganisms using 16S rRNA typing technique: The various microorganisms that were selectively accumulated in the gut of metal (Zn) treated earthworms were identified using 16Sr RNA sequencing technique. This was done by outsourcing to a reputed company.

Results and Discussion

Effect of exposure of earthworms to zinc: Zinc had a deleterious effect on growth and development of the earthworms. All the earthworms that were introduced in the soil spiked with zinc salt, irrespective of its concentration, showed variation in their weights by day3. The earthworms exposed to concentration of Zn more than 1600mg/kg were found dead within 6 days table-1.

Table-1
Effect of zinc on the earthworms *Pontoscolex corethrurus*

	Average weight & percentage weight gain/ loss on the days									
	0^{th}		3^{rd}		6^{th}		10^{th}		14^{th}	
Concentration of salt $ZnCl_2$ (mg/kg of dry wt. of soil)	g^a	$\%^b$	g^a	$\%^b$	g^a	$\%^b$	g^a	$\%^b$	g^a	$\%^b$
0	0.375(6)	100	0.490(6)	+30.6	0.574(6)	+53.0	0.436(6)	+16.2	0.498(6)	+32.8
100	0.326(6)	100	0.481(6)	+47.5	0.431(6)	+32.2	0.437(6)	+34.0	0.400(6)	+22.6
400	0.289(6)	100	0.324(5)	+12.1	0.345(5)	+19.3	0.318(5)	+10.0	0.313(5)	+0.08
800	0.205(6)	100	0.257(6)	+25.3	0.206(6)	0	0.205(6)	0	0.201(6)	0
1600	0.197(6)	100	0.191(6)	-0.03	0.212(5)	+0.07	0.212(5)	+0.07	0.197(5)	0
3200	0.362(6)	100	0.343(1)	-0.05	0	0	0	0	0	0
6400	0.248(6)	100	0	0	0	0	0	0	0	0
13200	0.217(6)	100	0	0	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 0^{th} day.

Table-2
Labeling of boxes and their corresponding amount of the $ZnCl_2$

Concentration of salt Zn Cl_2 (mg/kg dry wt. of soil)	Label
0	C Zn
100	Zn1
400	Zn2
1600	Zn3
3200	Zn4
6400	Zn5
13200	Zn6

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 in figure-1

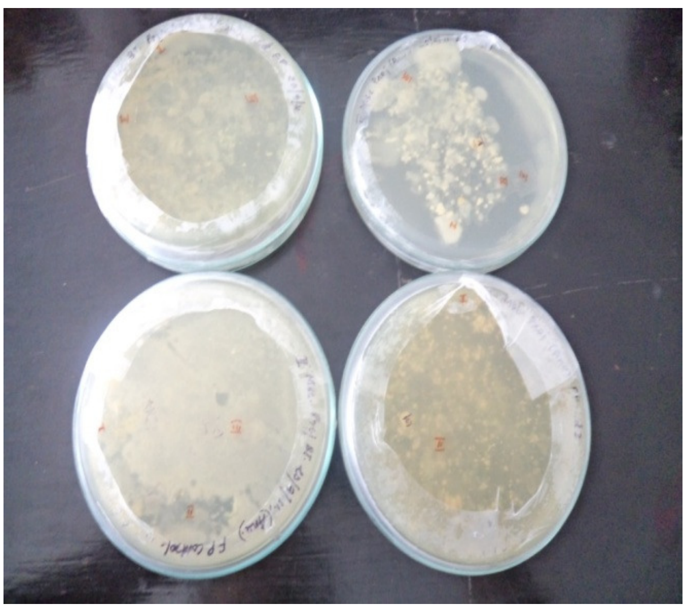

Figure-1
Colonies obtained after spread plating of the gut homogenate obtained from earthworms that survived the metal stress

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. 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. Based on the biochemical characterization of the isolated bacterial cultures they were identified as shown in table 3.

Table-3
Bacteria identified in the gut of the earthworms under zinc stress

Colony code[a]	Organism
ZnCFG	*B.cereus**
Zn1FG1	*B.cereus**
Zn1FG2	*N.mucosa*
Zn1FG3	*Aeromonas**
Zn2FG1	*B.cereus**
Zn2FG2	*Aeromonas**
Zn2FG3	*Aeromonas**
Zn3FG1	*Aeromonas**
Zn3FG2	*Aeromonas**
Zn3FG3	*Staphylococcus*
Zn4FG1	*B.cereus**
Zn4FG2	*Staphylococcus*
Zn4FG3	*Aeromonas**
ZnCMG1	*Aeromonas**
ZnCMG2	*Proteus*
ZnCMG3	*Aeromonas**
Zn1MG1	*Aeromonas**
Zn1MG2	*Aeromonas**
Zn1MG3	*N.mucosa*
Zn2MG1	*N.mucosa*
Zn2MG2	*Staphylococcus*
Zn2MG3	*Aeromonas**
Zn3MG1	*Aeromonas**
Zn3MG2	*B.cereus**
Zn3MG3	*N.mucosa*
Zn4MG1	*N.mucosa*
Zn4MG2	*B.cereus**
Zn4MG3	*Proteus*
ZnCHG1	*Aeromonas**
ZnCHG2	*Aeromonas**
ZnCHG3	*Proteus*
Zn1HG1	*Aeromonas**
Zn1HG2	*N.mucosa*
Zn1HG3	*Proteus*
Zn2HG1	*Aeromonas**
Zn2HG2	*Aeromonas**
Zn2HG3	*B.cereus**
Zn3HG1	*B.cereus**
Zn3HG2	*Aeromonas**
Zn3HG3	*Proteus*
Zn4HG1	*B.cereus**
Zn4HG2	*B.cereus**
Zn4HG3	*Aeromonas**

a: Colony ID : F-Foregut; M-Midgut; H-Hindgut; *: the organisms that were selectively accumulated under metal stress

As evident from the table-3, it is seen that a few of the microorganisms were retained only in specific regions of the gut. The bacteria specifically harbored in the gut of earthworms subjected to zinc stress were identified by comparing the microbes isolated from the gut of earthworms subjected to metal treatment with the gut micro flora of control earthworms. *Aeromonas hydrophila* and *Bacillus cereus* were found to be selectively accumulated in the gut of earthworms exposed to zinc stress.

Ribotyping of the selected microorganisms using 16S rRNA typing technique: The results of 16S ribotyping confirmed the identity of microorganisms harbored in the gut of zinc treated earthworms as *Aeromonas hydrophila* and *Bacillus cereus.*

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 zinc.

Some strains of Aeromonas are enteropathogens possessing a range of virulence factors and some are also resistant to heavy metals[13].A. hydrophila has broad metabolic capabilities including sulfate reduction and resistance mechanisms against toxic compounds encountered in polluted waters[14].Bacillus cereus has been reported to have tolerance to many heavy metals including nickel [15] and iron[16].

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.

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[17]. Apart from these chromosomal gene products, there are several microorganisms that perform detoxification with the aid of genes present on the plasmids.

Conclusion

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.

References

1. http://www.lenntech.com/periodic/elements/zn.htm#ixzz2pFELZB4i **(2014)**

2. Nies D.H., Microbial heavy-metal resistance, *Appl. Microbiol. Biotechnol.,* **51 (6)**, 730-50 **(1999)**

3. Reilly C., Metal Contamination of Food, London and New York: *Elsevier Science Publishers Lt*; 2nd ed. **(1991)**

4. http://www.lenntech.com/periodic/elements/zn.htm#ixzz2pFCnhTGm **(2014)**

5. Lavelle P., Melendez G., Pashanasi B. and Schaefer R., Nitrogen mineralization and reorganization in casts of the geophagous tropical earthworm *Pontoscolex corethrurus* (glossoscolecidae), *Biol. Fertil. Soils*., **14**, 49-53**(1992)**

6. Sturzenbaum S.R., Kille P. and Morgan A.J., Heavy metal-induced molecular responses in the earthworm, *Lumbricus rubellus* genetic finger printing by directed differential display, *Appl Soil Ecol.,* **9(3)**, 495-500 **(1999)**

7. Spurgeon D.J. and Hopkin S.P., Comparisons of metal accumulation and excretion kinetics in earthworms (Eisenia fetida) exposed to contaminated field and laboratory soils, *Appl Soil Ecol*., **11**, 227-243 **(1999)**

8. Dai J., Becquer T., Rouiller J.H., Reversat. G., Reversat F.B., Nahmani J. and Lavelle P., Heavy metal accumulation by two earthworm species and its relationship to total and DTPA-extractable metals in soils, *Soil Biol. Biochem*, **36**, 91–98 **(2004)**

9. Nahmani J., Hodson M.E and Black S., A review of studies performed to assess metal uptake by earthworms, *Environ. Pollut*., **145**, 402-424 **(2007)**

10. Andersen C. and Laursen J., Distribution of heavy metals in Lumbricus terrestris, Aporrectodea longa and A. rosea measured by atomic absorption and X-ray fluorescence spectrometry, *Pedobiologia.,* **24**, 347-356 **(1982)**

11. Kumar R., Singh B.L., Deepshikha V. and Shwetha., Enzyme activities and microflora of earthworm gut and vermireactors as indicators of the stabilization of waste degradation process, *Biorem. J*., **14(3)**, 150-157 **(2010)**

12. Cappuccino J. and Sherman N. Microbiology, A Laboratory Manual (7th Edition). Benjamin Cummings, 544 **(2004)**

13. Miranda C.D. and Castillo D., Resistance to antibiotic and heavy metals of motile aeromonads from Chilean freshwater, *Sci. Total Environ*., **224**, 167-176 **(1998)**

14. Seshadri R., Joseph S.W., Chopra A.K., Sha J., Shaw J., Graf J., Haft D.H., Wu M., Ren Q., Rosovitz M.J., Madupu R., Tallon L., Kim M., Jin S., Vuong H., Stine O.C., Ali A., Horneman A.J. and Heidelberg J.F., Genome sequence of Aeromonas hydrophila ATCC 7966T: Jack of all trades, *J. Bacteriol*., **188**, 8272-8282**(2006)**

15. Shoeb E., Ahmed N., Warner P.J., Morgan S. and Azim M.K., Identification of a unique mechanism of tolerance against nickel in Bacillus cereus from heavy metals contaminated sites, *The Internet journal of microbiology*, ISSN 1937-8289., **9(1), (2010)**

16. Harvie D.R., Vılchez S., Steggles J.R. and Ellar D.J., Bacillus cereus Fur regulates iron metabolism and is required for full virulence, *Microbiology.*, **151**, 569–577**(2005)**

17. Cobbett C. and Goldsbrough P., Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis, *Annu. Rev. Plant Biol*., **53**,159-182**(2002)**