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Multiple Metal Resistance in the Ciliate Protozoan, *Vorticella microstoma*, Isolated from Industrial Effluents and Its Potential in Bioremediation of Toxic Wastes

A. R. Shakoori, A. Rehman, Riaz-ul-Haq

Cell and Molecular Biology Laboratory, School of Biological Sciences, University of the Punjab, New Campus, Lahore 54590, Pakistan

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Aqueous effluents from mining, industries and different factories contain dissolved heavy metals, which may have an adverse impact on the environment if left untreated. Heavy metals have toxic effects on organisms: the characteristics and intensity of damage depends on the nature and level of the metal. Bacteria have been reported to tolerate heavy metal ions ranging from 10 to 100 mM and yeast ranging from 1 to 10 mM in a number of studies (Baldi et al.1990; Cervantes 1991; Gosh et al.1997; Ohtake et al. 1990; Yamamoto et al. 1993). Such high metal resistance has not been reported for Protozoa. However, protozoans have been found to be present in and metabolizing industrial effluents contaminated by toxic metal ions such as Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+} and Cd^{2+} and other toxic compounds (Schlenk and Moore 1994; Madoni et al.1996). The 1-hour LC_{50s} of Ni^{2+}, Hg^{2+}, Cd^{2+}, Pb^{2+} and Cu^{2+} for the protozoan *Euplotes mutabilis* have been reported to be 3.9, 1.0, 0.48, 0.37 and 0.29µg/mL, respectively (Al-Rasheid and Sleigh 1994). As regards Ciliophora, toxicity has been extensively studied in *Tetrahymena* and *Uronema* (Nilsson 1989).

Microorganisms can remove toxic metals from contaminated waters by converting them to forms that are precipitated from solution. In other instances, microbial alteration of the redox state of either the contaminant or the Fe^{3+} and Mn^{4+} oxides, which bind most heavy metals, can make metals more soluble, thus facilitating leeching of these contaminants from soil. Microorganisms with the ability to grow in the presence of heavy metals and with a significant metal uptake have a potential use in bioremediation of polluted waters. During this study a number of protozoans were observed in industrial effluents contaminated with Cr^{6+} amongst other heavy metals and chemical pollutants frequently used in tanning process. The ciliate protozoan, *Vorticella microstoma*, was observed to have high level of metal resistance. One important capability found in microorganisms, like bacteria, is the processing or detoxification of metal ions. This is achieved by reduction and oxidation reactions, adsorption, biosorption, and accumulation or binding with certain metal-binding proteins or metallothioneins (MTs). MTs are thought to be involved in metal homeostasis and detoxification, and scavenging of reactive oxygen species (Ghoshal and Jacob 2001). The present paper aims at determining ability of *V. microstoma* to reduce hexavalent chromium and processing of zinc, and to assess this property for its

Correspondence to: A. R. Shakoori

exploitation in metal detoxification and environmental bioremediation.

MATERIALS AND METHODS

Water samples were collected in screw capped sterile bottles, from Industrial waster water released by tanning industry near the city of Lahore (Pakistan). The bottles were half filled with the samples to allow air circulation. The pH and temperature of these samples were also recorded at the time of collection. Samples were inoculated in Bold-Basal salt medium (Haq et al. 1998) containing $NaNO_3$ (0.25g/L), $CaCl_2.2H_2O$ (0.025g/L), $MgSO_4.7H_2O$ (0.075g/L), K_2HPO_4 (0.075g/L), KH_2PO_4 (0.175g/L), NaCl (0.025g/L), EDTA (0.05g/L), KOH (0.031g/L), $FeSO_4.7H_2O$ (0.049g/L), H_2SO_4 (0.001,L/L), H_3BO_3 (0.01142g/L), $ZnSO_4.7H_2O$ (0.00881g/L), $MnCl_2.4H_2O$ (0.00144g/L), MoO_3 (0.00071g/L), $CuSO_4.5H_2O$ (0.00157g/L) and $Co(NO_3)_2.6H_2O$ (0.00049g/L). The pH of the medium was adjusted at 7.0.

The original sample of industrial wastewater contained a large number of bacteria, yeast, algae, and various protozoa. For isolation of protozoa, antibiotics, *i.e.* ampicillin (25µg/mL), chloramphenicol (50µg/ml) and gentamicin (10µg/mL), were added to the culture to prevent growth of bacteria. Algae were excluded by keeping the culture in semidarkness. Yeast was excluded by absence of any organic substance of the medium. Relatively pure culture of *V. microstoma* was prepared as follows. Several very small drops (5.0µL each) were taken from the sample and placed on a sterile slide. The drops were observed under a light microscope for the presence of *V. microstoma*. The drop having *V. microstoma* was selected and inoculated in the medium to get a pure culture of *V. microstoma*.

One hundred milliliter of Bold-Basal medium, in 250 mL conical flask, was inoculated under aseptic conditions with 10µL of inoculum containing 40-50 ciliates. Glucose as carbon source was added as 1g/L. The cultures were maintained in the laboratory for one week at room temperature (23 °C). Growth was observed in the culture by counting the number of protozoans at regular intervals.

For determination of cross heavy metal resistance of the Protozoa, the culture was aliquoted in five flasks. Same number of protozoa were maintained in all the cultures. Protozoan cultures were treated with four metal ions in separate 25ml conical flasks. For Cu^{2+} ($CuSO_4.5H_2O$), Cd^{2+} ($CdCl_2$), Zn^{2+} ($ZnSO_4$), and Cr^{6+} ($K_2Cr_2O_7$) the concentration in the medium on the first day was 5µg/mL with an increase of 5µg/ml Cu^{2+} every day for 44 days for Cu^{2+} and Cd^{2+}, 50 days for Zn^{2+}, and 52 days for Cr^{6+}. For treatment with Pb^{2+} [$Pb(NO_3)_2$] the concentration in the medium on the first day was 10µg/mL with an increase of 10µg/mL Pb^{2+} every day for 55 days. The initial concentration of metal ions and the daily increase in the concentration was based on the previous observations and the knowledge of relative toxicity of the metal ion to the microorganisms isolated from metal polluted industrial wastes. The metal treatment was stopped when the movement of the surviving protozoan cells came to a stand still. Although the

death of protozoa is confirmed by the lyses of the cells but the movement is considered to be a vital sign of life. When the protozoan became inactive, no more metal was added. Later it was observed that when ciliates were transferred to metal free media, movement was resumed. The control cultures with no metal added retained all the ciliary movements in *Vorticella.*

The number of *V. microstoma* in the cultures containing different metal ions was counted daily for 55 days. At least three counts were taken to get a mean of every reading (Haq et al. 1998). A control was run without addition of any metal ion. The activity, shape and size of the protozoans were also noted. The size was measured with an ocular micrometer after restricting the movement of the ciliates by putting the culture in methylcellulose and staining with 1% neutral red.

For determination of Cr^{6+} reduction by *V. microstoma*, Cr^{6+} was added to the culture at a concentration of 100μg/mL. The control culture medium contained Cr^{6+} at a concentration of 100μg/mL but was without ciliates. The cultures were incubated for 8 days and Cr^{6+} estimation was done by the diphenylcarbazide method every 48 hours (Chakrabarty and Mishra1992; Kunicka et al. 1992). A standard curve for the estimation of Cr^{6+} (1 to 100μg/mL) was prepared. For estimation of Cr^{6+}, a one mL culture was added to 49 mL distilled water in a beaker. The pH of the solution was adjusted to 0.8 by addition of H_2SO_4. The volume was made up to 100mL by addition of distilled water. A freshly prepared 2mL solution of diphenylcarbazide (5mg/mL in acetone) was added to the reaction mixture. The mixture was kept at room temperature for 5-10 minutes after which the optical density of the solution was taken at 540nm in a U-2000 Hitachi Spectrophotometer. The reduction in the amount of Cr^{6+} in the cultures was taken as the reducing ability of the protozoan.

For determination of Zn^{2+} reduction by *V. microstoma,* Zn^{2+} was added to the culture at a concentration of 100μg/mL. The control culture medium also contained Zn^{2+} at a concentration of 100μg/mL but was without the ciliates. The cultures were incubated for 8 days and from each medium (control and treated) 5mL culture was taken out under sterilized conditions after 48, 96, 144 and 192 hours, respectively. The cultures were spun down at 3000 rpm for 20 min and the supernatants were used for the estimation of Zn^{2+} by atomic absorption spectrophotometer (Varian, U.S.A) at wavelength 213.9nm. The amount of Zn^{2+} in the supernatants was determined using standard curve. The percentage reduction in the amount of Zn^{2+} in the medium was calculated.

Observations were made and all the experiments run in triplicate. At least three separate flasks were usually maintained for one treatment. The averages of control and experimental groups were compared and significant differences evaluated by using Student's 't' test of significance.

RESULTS AND DISCUSSION

The ciliate, *V. microstoma,* was found to be resistant to Cr^{6+} at a concentration of

260μg/mL (2.5mM). The Cr^{6+}-resistant ciliate was also found to tolerate Cu^{2+}, Pb^{2+}, Cd^{2+}, and Zn^{2+} at a concentration of 220μg/mL (3.4 mM), 550μg/mL (2.6 mM), 220μg/mL (2.0 mM), and 250μg/mL (3.8 mM), respectively. There was apparently no reduction in the size of *V. microstoma* cells. The cessation of movement of *V. microstoma* in different metal solutions was taken as a parameter of metal toxicity. The movements of ciliate slowed down in the presence of $K_2Cr_2O_7$ but completely stopped in the presence of $CuSO_4$. The presence of $CdCl_2$ decreased the ciliary movement, whereas the presence of $Pb(NO_3)_2$ and $ZnSO_4$ did not make any significant effect on the movement of ciliates. The order of resistance on the basis of motility was Pb^{2+}> Zn^{2+}> Cr^{6+}> Cd^{2+}> Cu^{2+}.

The level of heavy metal resistance in *V. microstoma* was very high as compared with the previous studies where it was only 4.5μM for Cu in *Euplotes mutabilis* (Al-Rasheid and Sleigh 1994). Cu at concentrations ranging from 1 to 10 decreased the total number of ciliates, as well as their diversity in activated sludge plants (Gracia et al. 1994). Similarly Pb was reported to lower total number of protist species in marine communities when added at a concentration of 1μg/mL (9.4μM). The protist biomass was reduced by 60% after the treatment (Fernandez and Novillo 1994). During present study a relatively high resistance of *V. microstoma* against five toxic heavy metal ions was noted. One of the salient features of the study was a long-term survival of the protozoan in the medium. The minimum time period for which the protozoan could tolerate heavy metal and the population starts decreasing was 44 days (for Cu^{2+}) and the maximum time for which the protozoan could tolerate heavy metal and after which the protozoan-population starts decreasing was 55 days.

The cell population, which is indicator of mitotic activity, has been adversely affected by the presence of metal ions in culture media. The number of the cells was reduced from 624 to 360 cell/mL in $K_2Cr_2O_7$, from 1230 to 836 cells/mL in $CuSO_4$, from 1540 to 935 cells in $Pb(NO_3)_2$, from 1085 to 690 cells in $CdCl_2$ and from 1320 to 880 cells in $ZnSO_4$ solutions. The reduction in the cell population was 42 % (Cr^{6+}), 32% (Cu^{2+}), 36% (Cd^{2+}), 43% (Pb^{2+}), and 41 % (Zn^{2+}), respectively. The order of resistance regarding the reduction in number of the cells was, therefore, Pb^{2+}> Cr^{6+}> Zn^{2+}> Cd^{2+}> Cu^{2+}. However, regarding molar concentrations of metal ions the order of resistance of *V. microstoma* against five metals was Zn^{2+}> Cu^{2+}> Pb^{2+}> Cr^{6+}> Cd^{2+}.

The ciliate showed remarkable ability to pick up heavy metals ions from the culture medium. The concentration of Cr^{6+} was reduced 36% after 48 hours, 40% after 96 hours, 45% after 144 hours and 48% after 192 hour in a culture medium containing Cr^{6+} (100μg/ml). On the other hand, the concentration of Zn^{2+} was reduced 80% after 48 hours, 96% after 96 hours, 98% after 144 hours and 99.2% after 192 hour when ciliates were grown in culture medium containing Zn^{2+} (100μg/ml). *V. microstoma* processed Zn^{2+} in the medium much more efficiently than it could process Cr^{6+}(Fig. 1).

Processing of metals by bacteria is documented (Ohtake et al. 1990; Yamamoto

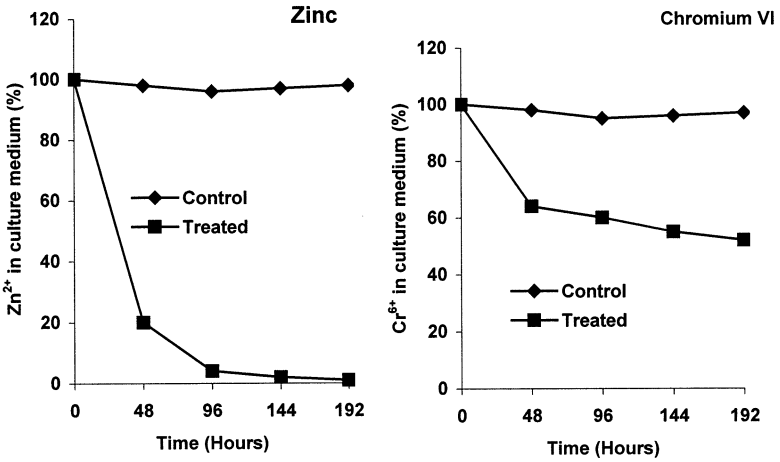

Figure 1. Reduction of zinc ions (Left) and chromium ions (right) from the culture medium containing potassium dichromate (100µg/ml Cr^{6+}) and zinc sulphate (100µg/ml Zn^{2+}) by *Vorticella microstoma*. The control cultures had the heavy metal ions, but no organisms.

et al. 1993) but not in Protozoa. The processing could be of two types: (1) bioaccumulation or (2) reduction. Bioaccumulation seems to be less feasible as it would have caused rapid death of the cells due to accumulation of the metal. Because of long time survival of the cells in the medium containing heavy metal, reduction as mechanism of heavy metal processing seems to be more feasible. Zn^{2+}, being an essential element involved in the synthesis of Chlorophyll and also a key component of many enzymes used in the processes of respiration and nitrogen metabolism, is probably adsorbed by the biomass, leading to reduction in concentration of zinc in the medium.

The isolation of heavy metal resistant protozoa from industrial effluents have shown that exposure to metal ions have induced efficient metal resistance, possibly as a result of adaptation or other genetic means. The study of metal resistance in protozoa may provide more clues about the processing of metal detoxification in mammalian cells. Moreover their frequent occurrence in wastewater or industrial effluents strongly indicates that they are able to withstand the heavy metal contaminated environment. This property makes protozoa excellent candidate for exploitation in metal detoxification and bioremediation.

It is concluded that *V. microstoma* is a suitable candidate for bioremediation of chromium- and zinc-contaminated industrial wastewater, as it can remove the heavy metals from the medium. The other components of microbial population (viz. bacteria, algae, fungi etc), however, will also have to be taken into consideration, while developing strategy for bioremediation (Haq and Shakoori 1998).

REFERENCES

Al-Rasheid KAS, Sleigh MA (1994) The effect of heavy metals on the feeding rate of *Euplotes mutablilis* (Tuffrau, 1960). European J Protistol 30: 270-279

Baldi E, Vaughan AM, Olson G L (1990) Chromium resistant yeast isolated form a sewage treatment plant receiving tannery wastes. Appl Environ Microbiol 56: 913-916

Cervantes C (1991) Bacterial interaction with chromate. Antonie-van Leeuwenhoek 59: 229-233

Chakrabarty AR, Mishra RK (1992) Speciation and determination of chromium in waters. Chem Speciat Bioavail 4: 131-134

Coppellotti O (1994) Effect of Cadmium on *Uronema marinum* (Ciliophora, Scuticociliatida) from Antarctica. Acta Protozool 33: 159- 167

Fernandez LG, Novillo A (1994) Effects of periodic addition of lead on a marine protistan community. Aquat Sci 56: 191-205

Gosh S, Mahapatra NR, Banerjee PC (1997) Metal resistance in *Acidocella* strains and plasmid mediated transfer of this characteristic to *Acidophylium multivorum* and *Escherichia coli*. Appl Environ Microbiol 63: 4523-4527

Gracia MP, Salvado H, Rius M, Amigo JM (1994) Effect of copper on ciliate communities from activated sludge plants. Acta Protozool 33: 219-226

Ghoshal K, Jacob ST (2001) Regulation of metallothionein gene expression. Prog Nucleic Acid Res Mol Biol 66: 357-384

Haq R, Shakoori AR (1998) Microbiological treatment of industrial wastes containing toxic chromium involving successive use of bacteria, yeast and algae. World J Microbiol Biotechnol 14: 583-585

Haq R, Qazi JI, Shakoori AR (1998) Growth and survival of protozoa isolated from a tannery effluent. Folia Microbiol 43: 109-112

Klaassen CD, Liu J, Choudhur S (1999) Metallothionein: an intracellular protein to protect against cadmium toxicity. Ann Rev Pharmacol 39: 267-294

Kojima Y, Binz PA, Jagi JHR (1999) Nomenclature of metallothionein: Proposal for a revision. In: Klaassen CD (ed) Metallothionein IV. Birkhauser Verlag Basel, pp 3-6

Kunicka T, Rinkis G, Ramane H (1992) A colorimetric method for chromium detection in biological materials. Latv Zinat Akad Vestis B Dala Dabasziant 10: 59-62

Madoni P , Davoli D, Gorbi G, Vescovi L (1996) Toxic effect of heavy metals on the activated sludge protozoan community. Water Res 30: 135-141

Nilsson JR (1989) *Tetrahymena* in cytotoxicology: with special reference to effects of heavy metals and selected drugs. European J Protistol 25: 2- 25

Ohtake H, Fuju E, Toda K (1990) Reduction of toxic chromate in an industrial effluent by use of a chromate reducing strain of *Enterobacter cloacae*. Envrion Technol 11: 663-668

Schlenk D, Moore CT (1994) Effect of pH and time on the acute toxicity of copper sulphate to the ciliate protozoan *Tetrahymena thermophila.* Bull Environ Contam Toxicol 53: 800- 804

Yamamoto K, Kato J, Yano, T, Ohtake H (1993) Kinetics and modeling of hexavalent chromium reduction in *Enterobacter cloacae.* Biotechnol Bioeng 41: 129-133