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Multiple Metal Resistance and Uptake by a Ciliate, *Stylonychia mytilus*, Isolated from Industrial Effluents and its Possible Use in Wastewater Treatment

A. Rehman · Farah R. Shakoori · A. R. Shakoori

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Environmental contamination with metals through industrial wastes is one of the major health concerns of developing countries. Metal pollutants can easily enter the food chain if heavy metal-contaminated soils are used for production of food crops. Farm productivity has been decreased in toxic metal polluted areas (Gosavi et al. 2004; Principi et al. 2006).

Chromium (Cr) is the seventh most abundant element on the earth crust (Katz and Salem 1994) and in waters originates from natural sources, such as weathering and rock constituents, wet precipitation and dry fallout from the atmosphere and runoffs from terrestrial ecosystem. However, chromium discharges from industries such as electroplating units, from leather tanning, metal finishing textile, nuclear power plants and paper industry increased its concentration in surface water several fold higher than its natural occurrence. Chromium exists in two oxidation states as Cr (III) and Cr (VI). The hexavalent form is 500 times more toxic than the trivalent form (Kowalski 1994). It is toxic to microorganisms, plants, animals and humans. Human toxicity includes lung cancer, as well as kidney, liver, and gastric damage (US Department of Health and Human Services 1991; Cieslak-Golonka 1995).

A. Rehman · F. R. Shakoori · A. R. Shakoori
Department of Microbiology and Molecular Genetics (AR) and School of Biological Sciences (ARS), University of the Punjab, New Campus, Lahore 54590, Pakistan

A. Rehman · F. R. Shakoori · A. R. Shakoori
Department of Zoology, GC University, Lahore, Pakistan

A. R. Shakoori (✉)
School of Biological Sciences , University of the Punjab, New Campus, Lahore 54590, Pakistan
e-mail: arshak@brain.net.pk

Lead contamination in surface water mainly comes from anthropogenic sources (96%), particularly from combustion of leaded fuels, pyrometallurgical non-ferrous metal production and coal combustion. Lead in natural waters may be in the form of organic lead complexes originally from the fuel of ever growing automobile population and subsequent break down of tetraethyl lead (Monterroso et al. 2003; Andrews and Sutherland 2004). The most serious effects of lead are related to impacts of central nervous system (Goyer 1993). It is considered a non-essential metal with no biological role in microorganisms, animals and plants (Bruins et al. 2000).

Microbial metal bioremediation is an efficient strategy due to its low cost, high efficiency and ecofriendly nature. Recent advances have been made in understanding metal–microbe interaction and their application for metal detoxification. Ciliate protozoa are cosmopolitan eukaryotic microorganisms adapted for life in soils and aquatic ecosystems. They are believed to be important grazers of bacteria and other microorganisms and in some artificial ecosystems such as activated sludge wastewater treatment plants, ciliates significantly improve effluent quality (Curds 1982; Nicolau et al. 2001).

One of the objectives of this study was to evaluate the survival of protozoa in media containing Cd^{2+}, Pb^{2+}, Cu^{2+} and Cr^{6+} and determine the uptake of lead and chromium by these organisms. A number of authors have already emphasized the role of protozoa in wastewater treatment plants (Fernandez-Leborans et al. 1998; Haq et al. 2000; Shakoori et al. 2004; Rehman et al. 2005).

Materials and Methods

Wastewater samples from a tannery effluent were collected in screw capped sterile bottles from Kasur (Pakistan). The

pH and temperature of these samples were recorded at the time of collection. The samples were inoculated in Bold-basal salt medium in 100 mL conical flasks (Haq et al. 1998). A large number of bacteria, yeast, algae, and various protozoa were present in the original wastewater sample.

For isolation of protozoa, antibiotics, i.e. ampicillin (25 μg/mL), chloramphenicol (35 μg/mL) and gentamicin (25 μg/mL), were used to prevent growth of bacteria. Algae were excluded by keeping the culture in semidarkness. Yeasts were excluded by absence of any organic substance in the medium. Cultures were plated onto YEPD medium (yeast extract 1 g, peptone 0.5 g, glucose 0.2 g in 100 mL distilled water pH 7.2–7.4; Sherman et al. 1986) and no growth appeared on the fungal medium (Shakoori et al. 2004; Rehman et al. 2005, 2006). Axenic cultures of protozoa were obtained according to Shakoori et al. (2004).

One hundred millilitres of Bold-basal salt medium [$NaNO_3$ (0.25 g/L), $CaCl_2.H_2O$ (0.025 g/L), $MgSO_4.7H_2O$ (0.075 g/L), K_2HPO_4 (0.075 g/L), KH_2PO_4 (0.175 g/L), NaCl (0.0025 g/L), EDTA (0.05 g/L), KOH (0.031 g/L), $FeSO_4.7H_2O$ (0.04 g/L), H_2SO_4 (0.001 L/L), H_3BO_3 (0.01142 g/L), $ZnSO_4.7H_2O$ (0.00881 g/L), $MnCl_2.4H_2O$ (0.00144 g/L), MoO_3 (0.00071 g/L), $CuSO_4.5H_2O$ (0.00157 g/L) and $Co(NO_3).6H_2O$ (0.00049 g/L)], diluted 1:1,000 with distilled water, in 250 mL conical flask, was inoculated under aseptic conditions with 10 μL of inoculum containing 40–50 ciliates. Glucose as carbon source was only added as 1 g/L in Bold-basal salt medium (Shakoori et al. 2004; Rehman et al. 2005, 2006). The cultures were maintained in the laboratory at room temperature (25–27°C). The pH of the medium was adjusted at 7.5. The growth of *Stylonychia* was observed in the cultures by counting the number of ciliates at regular intervals. Identification of the ciliates was done by observing their body shape, other morphological features, movements and behaviour (Edmondson 1966; Curds et al. 1983; APHA 1992).

Resistance of *Stylonychia* to four metal ions, i.e. Cr^{6+}, Cu^{2+}, Pb^{2+} and Cd^{2+} was checked by addition of the respective metal salts ($K_2Cr_2O_7$, $CuSO_4.5H_2O$, Pb $(NO_3)_2$ and $CdCl_2$) to Bold-basal salt medium. Metals ions were sterilized separately and added to the medium when the temperature of the salt medium was slightly less than 50°C. For Cr^{6+}, Cu^{2+}, and Cd^{2+} the concentration in the medium on the first day was 1 μg/mL with an increase of 1 μg/mL every day for 30 days for Cr^{6+}, 20 days for Cu^{2+}, and 23 days for Cd^{2+}. For treatment with Pb^{2+} the concentration in the medium on the first day was 2 μg/mL of Pb^{2+} with an increase of 2 μg/mL of Pb^{2+} every day for 30 days. Although the death of protozoa is confirmed by the lysis of the cell, movement is considered to be a vital sign of life. When the protozoa became inactive, no more metal was added.

The effect of different metal ions on the growth of culture was checked by counting the number of protozoan cells in the medium. The cells were grown in the salt medium, to which lead or chromium was added at a concentration of 1 μg/mL per day for 8 days. At least three counts were taken every day to get a mean of every reading. The growth was compared with that of the control culture, which contained no added metal ions. 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 determining uptake of heavy metals by *Stylonychia mytilus*, the ciliates were grown by inoculating 100 mL of Bold-basal medium, in four 250 mL conical flasks, with 10 μL of original laboratory culture (40 ± 2 cells) at 25°C for 3 days. On fourth day, when the culture had $1.2 \pm 0.2 \times 10^3$ cells/mL, two culture flasks were autoclaved to kill the ciliates, whereas ciliates were kept alive in the other two flasks. After that chromium was added in the two flasks (one with live and the other with killed ciliates) and likewise lead was added in the remaining two flasks (one with live and the other with killed ciliates) at a concentration of 10.0 μg/mL of each metal. The cultures were incubated for 6 days and from each flask 5 mL culture was taken out under sterile conditions after 0, 48, 72, 96 h, respectively. Two control flasks were kept in parallel for each metal, but without any organism. The cultures were spun down at 3,000 rpm for 15 min and the supernatants were used for the estimation of Pb^{2+} and Cr^{6+} by atomic absorption spectrophotometer (Varian, USA) at wavelength 217.0 and 357.9 nm, respectively. The amount of metals in the supernatants was determined using standard curves. The percentage reduction in the amount of Pb^{2+} and Cr^{6+} in the medium was calculated and was directly related to corresponding uptake by the organisms.

For further confirmation of metal uptake by the protozoans, the ciliates were grown in two 250 mL conical flasks containing 100 mL of Boald-basal medium, to which Pb^{2+} and Cr^{6+} (10 μg/mL) were added separately and incubated at 25°C. After 96 h the cells were pelleted, washed three times in saline solution and acid digested (H_2SO_4:HNO_3, 1:1). Each metal contents of the digest were measured by Atomic Absorption Spectrophotometer (AAS) at their respective wavelengths. All the experiments were done in triplicate. Amount of chromium and lead uptake by ciliate cells was calculated in μg/mL by using standard curve.

All values are average of three readings and have been shown as mean ± SEM. For determining significance of differences between the control and the experimental, Student' "*t*" test was applied.

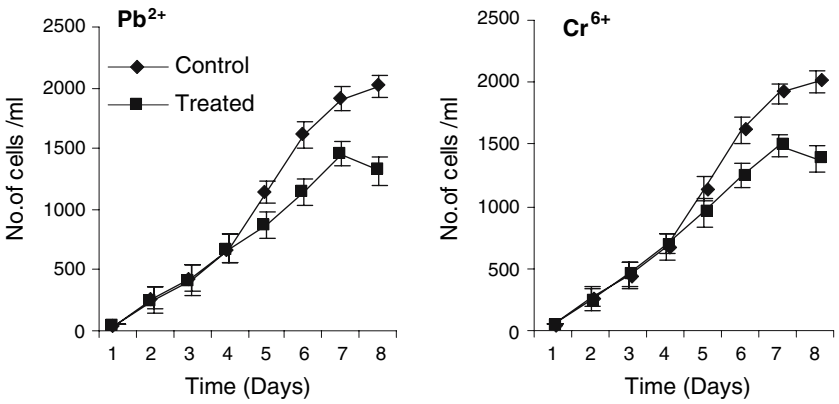

Fig. 1 Growth curves of *Stylonychia mytilus* in $Pb^{2+}$and Cr^{6+} containing medium. Control culture did not contain any metal ions

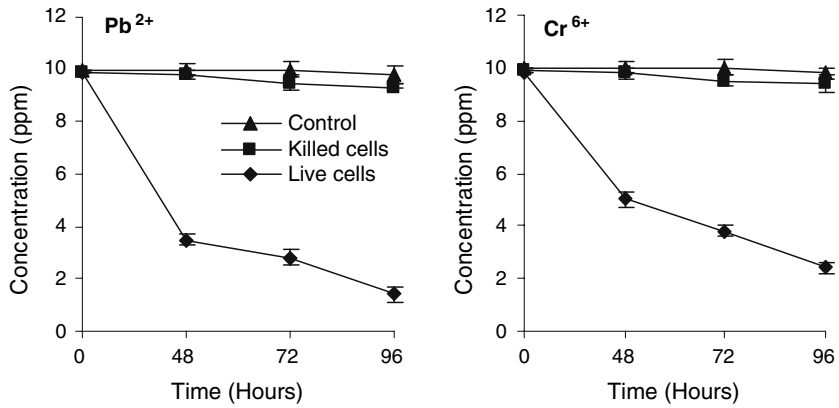

Fig. 2 Uptake of Pb^{2+} and Cr^{6+} by *Stylonychia mytilus* (live and killed cells) growing in Pb^{2+} and Cr^{6+} containing medium. The control did not contain cells of the isolate

Results and Discussion

Figure 1 shows growth curves of *S. mytilus* in a medium with and without metal ions. The growth of ciliate, which is indicated by cell population, has been affected by the presence of metal ions in culture media. The control culture of *S. mytilus* contained 0.058×10^3 cells/mL on day 1, which increased to 2.008×10^3 cells/mL after 8 days. However, when Cr^{6+} (8 μg/mL) was added the number increased from 0.050×10^3 to 1.383×10^3 cells/mL. In the presence of Pb^{2+} the number of cells increased from 0.058×10^3 to 1.317×10^3 cells/mL after 8 days.

Stylonychia mytilus was found to resist Pb^{2+} up to a concentration of 60 μg/mL. The Pb-resistant ciliate could also tolerate Cu^{2+}, Cr^{6+} and Cd^{2+} at the maximum concentrations of 20 , 30 and 23 μg/mL, respectively. There was apparently no reduction in the size of *S. mytilus* cells. Movement, which is a vital sign of life, was taken as a parameter of metal toxicity. The movements of the ciliate slowed down in the presence of $K_2Cr_2O_7$ (30 μg/mL) but almost stopped in the $CuSO_4$ (20 μg/mL) and $CdCl_2$ (23 μg/mL). The presence of Pb $(NO_3)_2$ (60 μg/mL) did not have any significant effect on the movement of ciliates. The order of resistance on the basis of motility was $Pb^{2+} > Cr^{6+} > Cd^{2+} > Cu^{2+}$. Metal resistant protozoa have been reported in wastewaters and metal-polluted environments (Madoni et al. 1996; Shakoori et al. 2004; Rehman et al. 2005; Madoni and Romeo 2006).

Metal uptake phenomenon includes both passive adsorption of heavy metals to the cell walls and metabolically mediated uptake (Gadd 1990). In the present study the metal was removed by one or more of these processes. Figure 2 shows the removal of heavy metal ions from the medium by live and killed ciliates. The live *S. mytilus* growing in medium containing lead (10.0 μg/mL) could reduce 80% (867 cells/mL) of lead from the medium after 48 h, 84% (1,142 cells/mL) after 72 h and 88% (1,458 cells/mL) after 96 h, respectively. Likewise live ciliate reduced 52% (700 cells/mL) chromium from the medium after 48 h, 76% (1,250 cells/mL) after 72 h and 80% (1,492 cells/mL) after 96 h, respectively (Fig. 2). This clearly indicates that the ciliates actively take up the heavy metals. Metal bioaccumulation has also been reported to be the main mechanism of resistance to heavy metals in ciliates by others (Martin-Gonzalez et al. 2006; Diaz et al. 2006).

It is well recognized that microorganisms have a high affinity for metals and can accumulate metals by a variety of mechanisms (Pas et al. 2004; Jeyasingh and Philip 2005; Harrison et al. 2006). These have been used to remove metals from polluted industrial and domestic effluents on a large scale. Microorganisms have a high surface area-to-

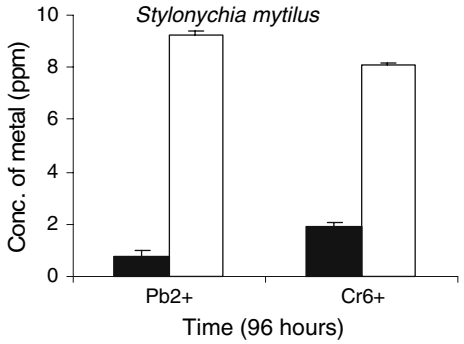

Fig. 3 Amount of lead and chromium accumulated by *Stylonychia mytilus* (pellet *open square*) and in the medium (supernatant *filled square*) after 96 h of incubation

volume ratio because of their small size and therefore provide a large contact area that can interact with metals in the surrounding environment (Ledin 2000).

Shakoori et al. (2004) reported that *Vorticella microstoma* showed remarkable ability to pick up metal ions from the culture medium. The concentration of Zn^{2+} and Cr^{6+} was reduced 99% and 48% after 192 h, respectively. Mortuza et al. (2005) reported that *Paramecium bursaria* accumulated 1.72 to 15.5 pg Cr/cell in a time and concentration–dependent manner. In the present investigation *S. mytilus* accumulated 5.4 pg Cr and 6.3 pg Pb/cell from the medium after 96 h of incubation (Fig. 3). These microorganisms actively contribute to the amelioration of the effluent quality, since the majority of them feed upon dispersed bacteria (Madoni 2000).

Ciliates having the potential to detoxify metals described in this study have become extremely important for microbiological detoxification of polluted water because of the consistently deteriorating environmental situation in developing countries like Pakistan. Conventional methods for the treatment of metals include chemical reduction by using a reducing agent such as sodium sulfate and adsorption on the ion exchange and chelating resins. However, these methods consume high amounts of energy and large quantities of chemical reagents. It is well known that bioremediation of toxic pollutants has advantages over other techniques as it is cheap, non-destructive and contamination remains localized (Eccles 1995; Rise-Roberts 1998).

Uptake of metals by living cells has become one of the most attractive means for bioremediation of industrial wastes and other metal polluted environments. Metal uptake processes by biological cells are known under the general term of biosorption. These phenomena include both passive adsorption to the cell walls and metabolically mediated uptake by the cells (Gadd 1990). In one of the previous reports from this laboratory *Euplotes mutabilis* grown in the medium containing Cu^{2+} (5 μg/mL) has been reported to reduce 60% of copper from the medium after 48 h, 82% after 72 h and 95% after 96 h (Rehman et al. 2006). It could also reduce 67% Hg^{2+} after 48 h, 75% after 72 h, and 82% after 96 h from the medium containing Hg^{2+} at a concentration of 10 μg/mL. Likewise, *Stylonychia* has also been reported to actively take up Pb^{2+} from the medium. The protozoan culture grown in medium containing lead (10 μg/mL) could reduce 80% of lead from the medium after 48 h, 82% after 72 h and 86% after 96 h, respectively (Rehman et al. 2005). In the present study live *S. mytilus* could remove 88% Pb^{2+} and 80% Cr^{6+} from the medium after 96 h of incubation, whereas killed organisms could remove only negligible quantity of metal from the medium. This finding conclusively indicates uptake of metals by the ciliate. Hence, there is a possibility of the use of *S. mytilus* as a biomonitor and bioaccumulator for lead and chromium contaminants in aquatic environments.

In this study we have reported the isolation of *S. mytilus* which is resistant to highly toxic metal ions and may be employed for metal detoxification operations.

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