Detoxification of toxic heavy metals by marine bacteria highly resistant to mercury.
De Jaysankar, N. Ramaiah and L. Vardanyan. Marine Biotechnology. Vol. 10, No. 4, pp. 471-477, 2008.

Detoxification of toxic heavy metals by marine bacteria highly resistant to mercury

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Running title: Detoxification of toxic heavy metals by marine BHRM

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Abstract

Pollution in industrial areas is of serious environmental concern and interest in bacterial resistance to heavy metals is of practical significance. Mercury (Hg), Cadmium (Cd) and lead (Pb) are known to cause damage to living organisms including human beings. Several marine bacteria highly resistant to mercury (BHRM) capable of growing at 25 ppm (mg L^{-1}) or higher concentrations of mercury were tested during this study to evaluate their potential to detoxify Cd and Pb. Results indicate their potential of detoxification not only of Hg, but also Cd and Pb. Through biochemical and 16S rRNA gene sequence analyses, these bacteria were identified to belong to *Alcaligenes faecalis* (seven isolates), *Bacillus pumilus* (three isolates), *Bacillus* sp. (one isolate) *Pseudomonas aeruginosa* (one isolate) and *Brevibacterium iodinium* (one isolate). The mechanisms of heavy metal detoxification were through volatilization (for Hg), putative entrapment in the extracellular polymeric substance (for Hg, Cd and Pb) as revealed by the scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS), and/or precipitation as sulfide (for Pb). These bacteria removed over 70% of Cd and 98% of Pb within 72 and 96 h respectively from growth medium that had initial metal concentrations of 100 ppm. Their detoxification efficiency for Hg, Cd and Pb indicates good potential for application in bioremediation of toxic heavy metals.

Keywords: Heavy metals, bacteria highly resistant to mercury, detoxification, volatilization, *mer*A, energy dispersive X-ray spectroscopy, scanning electron microscopy, bioremediation

INTRODUCTION

Metal-resistant microorganisms have been isolated from polluted environments and studies on the interactions between heavy metals and microorganisms have focused on bacterial transformation and conversion of metallic ions by reduction (Chang et al., 1993). Metal-resistant microorganisms may be useful as indicators of potential toxicity to other forms of life (Doelman et al., 1984) and are important in studies of mechanisms, determinants and genetic transfer of microbial metal-resistance (De Rore et al., 1994). Hg^{2+}, Pb^{2+} and Cd^{2+} are of serious concern as they are non-biodegradable, highly toxic and are present in a variety of waste streams that contaminate the environment. These three metals are included on the US Environmental Protection Agency's list of priority pollutants (Cameron, 1992).

Bioremediation processes are very attractive in comparison with physicochemical methods for heavy metal removal because they can have lower cost and higher efficiency at low metal concentrations (Gadd and White, 1993). Mechanisms of metal resistance in microbes include precipitation of metals as phosphates, carbonates and/or sulfides; volatilization via methylation or ethylation; physical exclusion of electronegative components in membranes and extracellular polymeric substances (EPS); energy-dependent metal efflux systems; and intracellular sequestration with low molecular weight, cysteine-rich proteins (Gadd, 1990; Silver, 1996).

The *mer* operon that confers mercury resistance to bacteria is widely distributed in mercury-resistant bacterial populations (Osborn et al., 1997; Barkay et al., 2003) and is fairly highly conserved. In contrast, other metal resistance systems have evolved several times (Silver and Phung, 1996). Single bacterial strains can be resistant to many metals. The genome sequence of *P. putida* KT2440 contains 61 open reading frames involved in tolerance and resistance to a range of metals (Cànovas et al., 2003). Resistance to multiple metals including Hg, Cd, Zn, Sn, Cu, and Pb was found in a bacterium isolated on the basis of tributyltin resistance (Pain and Cooney, 1998).

The present study focuses on 13 marine bacterial strains that are highly resistant to mercury (De et al., 2003) and investigates their resistance and ability to detoxify Cd and Pb in addition to Hg.

Since Hg, Cd and Pb are present in many environments, the remediation of these metals is of great ecological interest.

MATERIALS AND METHODS

Bacterial isolates. Mercury-resistant bacteria were isolated from various locations along the Indian Coast. Seawater nutrient agar (SWNA, Himedia Laboratories Pvt. Ltd., India; formula per liter: peptone 5.0 g, beef extract 1.5 g, yeast extract 1.5 g, sodium chloride 15 g, aged seawater 500 ml, deionized water 500 ml and agar 15 g), amended with 10 ppm $HgCl_2$ (ca. 50 mM Hg)(Merck, Germany) was used for their isolation and enumeration. Bacterial colonies capable of growth when streaked onto SWNA plates with 25 ppm mercury were termed bacteria highly resistant to mercury (BHRM). Purified cultures of 13 BHRM isolates were previously grown in seawater nutrient broth (SWNB: SWNA without agar) amended with 25 ppm Hg, characterized and identified using biochemical tests and 16S rDNA sequencing (De et al., 2007).

Volatilization of Hg. Details of this experiment is already published (De et al., 2006). In brief, 11 BHRM, six isolates of *Alcaligenes faecalis* (GP15, GO02, GP16, GP06, GP14, GP17), three isolates of *Bacillus pumilus* (CM10, CH13, GP08), one *P. aeruginosa* isolate (CH07), one *Brevibacterium iodinium* isolate (GP13), one mercury-sensitive bacterium (unidentified; as negative control) and *Pseudomonas putida* KT2442::mer73 (as positive control) were tested. Strains were grown in marine broth 2216 (Difco). Cells were pelletted by centrifugation (at 10,000 X g for 10 minutes), washed with phosphate buffer (pH 7) and transferred to microtitre plates. Mercurated phosphate buffer (10 ppm Hg final concentration) was added to the cells. The plate was covered with Kodak XAR film and incubated at 30ºC in the dark for 4 hours, prior to removal and development of the film.

Detection of merA. Genomic DNA was extracted from cultures grown for 18 h using the NucleoSpin Extract Kit (Macherey Nagel, Germany). The *merA* region was amplified using primer pairs (A1 forward: 5′ACC ATC GGC GGC ACC TGC GT3′; A5 reverse: 5′ACC ATC GTC AGG TAG GGG ACC AA3′) synthesized by Invitrogen (Invitrogen GmbH, Germany). The PCR was performed (Mastercycler gradient, Eppendorf, Hamburg, Germany) using predenaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 15 sec,

annealing at 58°C for 30 sec, extension at 68°C for 1 min and a final extension at 68°C for 4 min. The PCR products were visualized by agarose gel electrophoresis.

Detoxification of Cd. Two isolates (CH07, GP06) were used in the experiment with Cd. A Cd sensitive *Proteus* sp. isolate CH05 and heat killed bacterial cells were included as negative controls. Bacteria were tested in SWNB amended with different concentrations of Cd (as $CdCl_2$) at room temperature (28±2ºC). Sub-samples (1 ml) were taken every 24 h, centrifuged at 10,000 X g for 15 minutes at 24ºC and supernatants filtered through sterilized pre-weighed 0.22 µm membrane filters. Filtrates were diluted 10-fold with 10% HNO_3 for estimation of the heavy metals from the test media. The pellets were digested overnight in 1 M HCl, sonicated twice for 45 seconds, and centrifuged at 8,000 X g for 5 min. The supernatants were collected and diluted with 10% HNO_3 for estimation of heavy metals accumulated by the cells. The cell pellets were dried for at least 48 hour at 70ºC and weighed to determine bacterial biomass. The Cd concentrations were determined by inductively coupled plasma-atomic emission spectrometry (Varian, Liberty Series II AX, Sequential ICP-AES) under the following conditions (pump rate: 15 rpm, plasma gas: 15.0 L min^{-1}, fast pump: on, PMT voltage: 650V, auxiliary gas: 1.50 L min^{-1}, rinse time: 10 sec, sample uptake: 30 sec, wavelength: 226.502 nm, replicate no. 3). The metal concentrations were calculated using appropriate blanks (growth medium diluted with 10% HNO_3) and standards.

Detoxification of Pb. Three BHRM isolates (CH07, GP13, and S3) were tested in SWNB amended with different concentrations of Pb [as $(CH_3COO)_2Pb$] at room temperature (28±2ºC). Mercury-sensitive isolate CH05 and heat-killed bacterial cells were included as negative controls. The experimental procedure was similar to that for Cd. Atomic absorption spectrophotometry (Perkin Elmer, Analyst 200 for flame analysis) under the following conditions (slit width: 1.0 nm, lamp current 8.0.mA, scale expansion: 1.000, wavelength: 217 nm, atomization: air-acetylene, integration time: 3.0 sec, replicate no. 3) was carried out for determination of Pb concentrations, using appropriate blanks and standards for calibration.

Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDS). Samples (10 ml) of broth cultures from test flasks with Cd and Pb were centrifuged at 8,000 X *g* at 4ºC for 5 minutes. The cells were washed twice with 0.1 M phosphate buffered saline (PBS;

15 mM phosphate buffer, 138 mM NaCl, 2.7 mM KCl, pH 7.4 at 25ºC) and fixed overnight in 2% glutaraldehyde (prepared in 0.1 M PBS). The cells were washed with PBS and distilled water prior to dehydration through an ethanol series (10% to absolute), held at each concentration for 30 minutes. Samples were placed on a brass stub, sputter-coated with gold and examined by SEM (SEM-JEOL-JSM5800LV). EDS (OXFORD ISIS 300 EDS) was carried out to detect Cd and Pb and their compounds that were either adsorbed to the cell surface or entrapped in the exopolymeric substance (EPS).

RESULTS

Bacterial isolates. 16S rRNA gene sequence analysis showed that the BHRM isolates are closely related to *A. faecalis* (GO01, GO02, GP06, GP14, GP15, GP16 and GP17), *B. pumilus* (GP08, CH13 and S3), *P. aeruginosa* (CH07), *B. iodinium* (GP13) and *Bacillus* sp. (CM10). The partial 16S rRNA gene sequences have been deposited in the Gene Bank database under accession numbers DQ37744-DQ377468 (De et al., 2007).

Volatilization of Hg. All of the 11 BHRM were capable of volatilizing mercury indicated by fogging of the XAR film covering their respective wells due to the reduction of $AgCl_2$ by gaseous Hg. The negative control did not volatilize mercury indicated by clear film over this isolate.

Presence of *merA* genes. PCR analyses revealed the presence of putative *merA* gene fragments in nine isolates: GO02, GP06, GP08, GP14, GP15, GP16, GP17, CH07 and GO01. All these isolates volatilized mercury. Putative *merA* genes were not detected in isolates CH13 and CM10 that were shown to volatilize Hg. The mechanism of this apparently non-*mer*A mediated volatilization is not known.

Detoxification of Cd. Isolate CH07 removed Cd from the medium at a faster rate than GP06 during the first 48 hours (Figures 1A-B). The Cd concentration was reduced from an initial concentration of 100 ppm to 17.4 ppm by CH07 and to 19.2 ppm by GP06 in about 72 h. This was consistent with an increase in Cd concentration in the cell pellets (Figures 2A-B), Isolates CH07 and GP06 removed over 75% and 70% of Cd respectively from the growth medium. In

controls containing killed cells, removal of metals was <5%. The metal-sensitive control isolate CH05 showed no sign of growth or Cd accumulation (Figure 1C).

Detoxification of Pb. All three BHRM isolates tested were able to remove Pb from the growth medium (Figures 3A-C). In case of CH07, the concentration of Pb was reduced to 1.8 ppm (>98% removal) from an initial concentration of 100 ppm in 96 h. SEM and EDS analysis (below) indicated that Pb was likely present in the EPS. Isolates GP13 and S3 removed more than 87 and 88% of Pb respectively from the growth medium. The fact that these isolates were capable of hydrogen sulfide production and indications from preliminary chemical tests and EDS, suggest that Pb was precipitated as sulfides by these two strains. The metal-sensitive negative control isolate CH05 showed no sign of growth (Figure 3D).

SEM and EDS. The morphology of isolate GP13 was unchanged after growth in medium without added Pb ((Figure 4a) and with 50 ppm Pb (Figure 4b). This suggests that Pb is not toxic to the isolate under the conditions tested. Preliminary studies showed that the Pb-resistant bacteria produced large quantities of EPS. EDS analysis indicated that Pb was most likely entrapped in the EPS in case of CH07. Preliminary chemical tests showed that Pb was most likely precipitated as sulfide by isolates GP13 and S3. EDS analysis of the cells showed the concentrations of Pb and Cd to be as high as 21% and 19%, respectively. Bacteria grown in medium without these heavy metals showed no EDS signals for either Cd or Pb.

DISCUSSION

Bacterial mobile genetic elements, such as plasmids or transposons, can carry multiple genes encoding metal and antibiotic resistance. Thus, exposure to one agent may select for microorganisms resistant to several toxicants. Such organisms may be important in performing biological processes in contaminated habitats. Metal-resistant strains may also have application in remediation of metal-contaminated environments. A genetically engineered *Escherichia coli* strain with Hg^{2+} transport system and metallothionein has been used to bioaccumulate mercury from wastewater (Deng and Wilson, 2001). *Deinococcus radiodurans* has been engineered to remediate radioactive mixed waste as well as vaporize mercury (Brim et al., 2000).

Previous studies in terrestrial and freshwater environments have shown that as many as 100% culturable mercury-resistant environmental isolates contain genes that have homology to either *merTn21* (Barkay et al., 1989) or *merTn501* (Bruce et al., 1995). However, there are conflicting findings on the frequency of prototypic *mer* genes in culturable mercury-resistant isolates obtained from marine samples (Rasmussen and Sørensen, 1998; Reyes et al., 1999). In this study, only 9 of the 11 BHRM isolates were positive for *merA*, whereas all of them volatilized Hg^{2+} to Hg^{0}. This may suggest the presence of putative non-*mer* mediated mercury volatilization in two isolates but this interesting possibility remains to be confirmed.

The reduction of Cd in culture supernatants and accumulation in cell pellets of bacteria found in this study is consistent with microbial bioabsorption of the metal (Nies, 1999). Several mechanisms of Cd resistance have been described (Lee et al., 2001) and may be responsible for Cd resistance in these marine BHRM. Previously, *P. aeruginosa* CW-96-1 was reported to grow aerobically at 5 mM Cd (Wang et al., 1997) and *P. aeruginosa* PU21 (Rip64) accumulated 58 mg Cd g^{-1} dry wt (Chang et al., 1997). *Klebsiealla planticola* (Cd-1) grew anaerobically at a Cd concentration of 15 mM and precipitated CdS (Sharma et al., 2000). Bang et al. (2000) achieved 91% removal of Cd from an initial concentration of 200 μM Cd by using a strain of *E. coli*. Highly resistant strains of *Bacillus* H9 and *Pseudomonas* H1 tolerated up to 275 and 225 μg ml^{-1} of soluble Cd, respectively (Roane and Pepper, 2000).

P. aeruginosa PU21 (Rip64) resting cells were reported to take up as much as 110 mg Pb g^{-1} dry cell mass whereas inactivated cells absorbed 70 mg Pb g^{-1} dry cell mass (Chang et al., 1997). This bacterium could adsorb Hg^{2+} up to 400 mg Hg g^{-1} dry cell mass. Cysteine-rich transport proteins associated with the cell membrane were postulated to be important in metal adsorption in this bacterium.

SEM and EDS analyses suggest that Pb was entrapped in EPS in the case of isolate CH07. It is likely that Pb was precipitated as sulfide by isolates GP13 and S3. In all cases in our study, dead cells accumulated only very small amount of heavy metals, suggesting that the BHRM possess resistance mechanisms for toxic heavy metals and are capable of active removal of metals from the surrounding medium. Negatively charged EPS could bind lead and prevent its entry into the

cell in case of CH07 and a similar resistance mechanism has been reported for mercury (Dong et al., 2000).

Resistance to multiple metals has been found in several other bacterial systems and characterized at the molecular level. Liesegang et al. (1993) reported that *A. eutrophus* CH34 harbors numerous heavy metal resistance determinants including three for mercury resistance, one for chromate resistance and two for divalent cations, called *czc* (for Cd^{2+}, Zn^{2+} and Co^{2+}) and *cnr* (for Co^{2+} and Ni^{2+}). *Czc* is an efflux pump that functions as a chemio-osmotic divalent cation/proton antiporter (Nies and Silver, 1995). Homologues of the *czc* genes, called *czr*, which confer resistance to Cd and Zn, have also been identified in *P. aeruginosa* (Hassan et al., 1999). Mutational analysis indicated that *cad*A and *cad*R are fully responsible for cadmium resistance and partially for zinc resistance (Lee et al., 2001). It is quite likely that the multi-metal resistant BHRM possess the genetic components for dealing with many toxic metal ions. These isolates are of interest for molecular characterization of mechanisms for resistance to multiple metals and and hold promise for bioremediation of toxic heavy metals, including in environments that are contaminated by several metals.

ACKNOWLEDGEMENTS

We thank the Director of the NIO for facilities and encouragement. We thank Drs. P. V. Narvekar and M. S. Prasad, Mrs. A. Mesquita, Mr. B. Fernandez and Mr. Khedekar for their suggestion and technical assistance. We thank all the anonymous reviewers and Dr. Russell Hill at the Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, Md. for their help in improving the ms. CSIR-SRF grant 31/26/75/2002 EMR-I and UGC-DAAD short-term scholarship to De J are gratefully acknowledged. This is NIO contribution number xxx.

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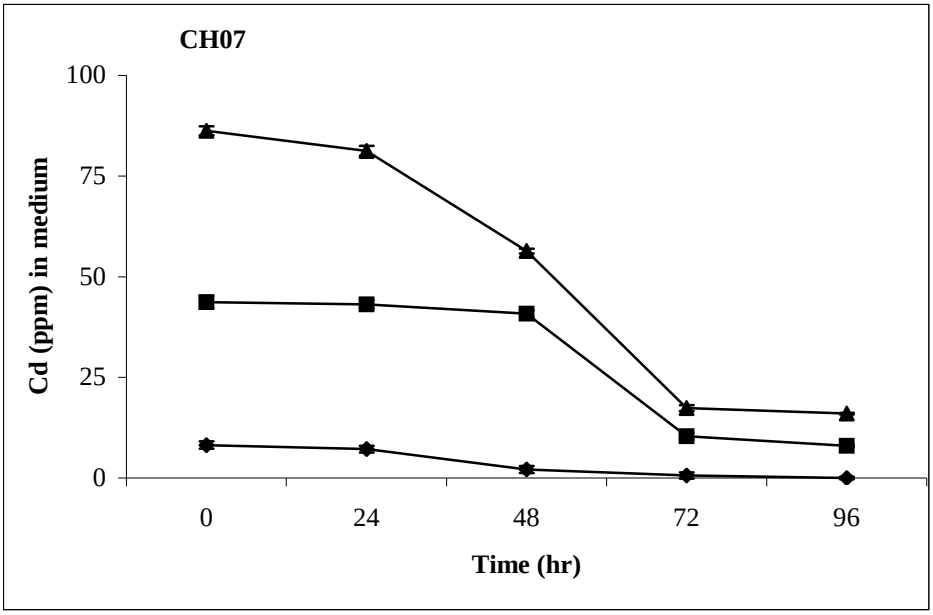
LEGENDS TO FIGURES

Figures 1A-C. Cd removal by BHRM isolates CH07 (A) and GP06 (B) bacteria from the supernatant of medium SWNB amended with different concentrations of Cd, compared with metal-sensitive control isolate CH05 (C).

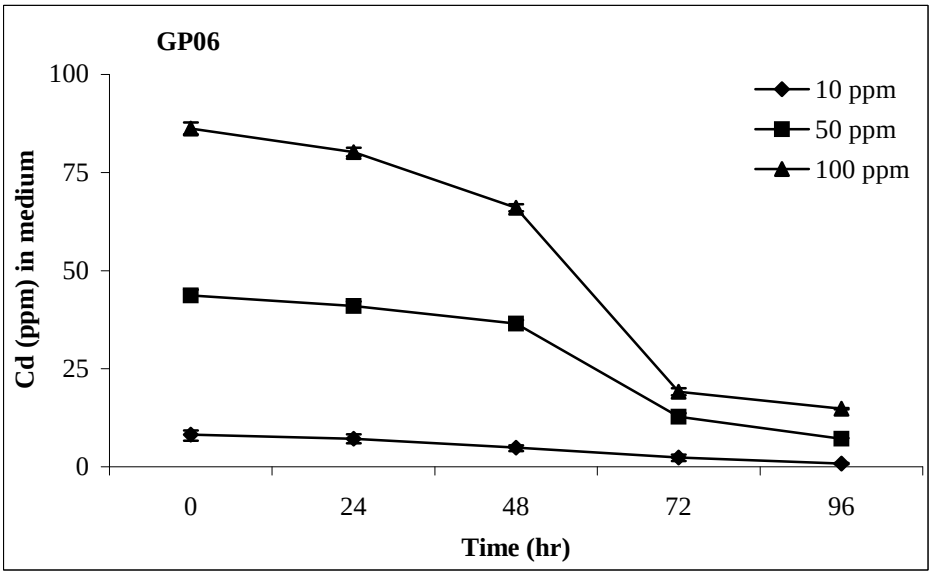
Figures 2A-B. Cd concentrations (μg g^{-1} dry wt. h^{-1}) in cell pellets of BHRM isolates CH07 (A) and GP06 (B) grown in SWNB amended with different concentrations of Cd.

Figures 3A-D. Removal of lead from medium by BHRM isolates when grown in seawater nutrient broth amended with different concentrations of Pb. A., CH07; B., S3; C., GP13; and D., negative control CH05

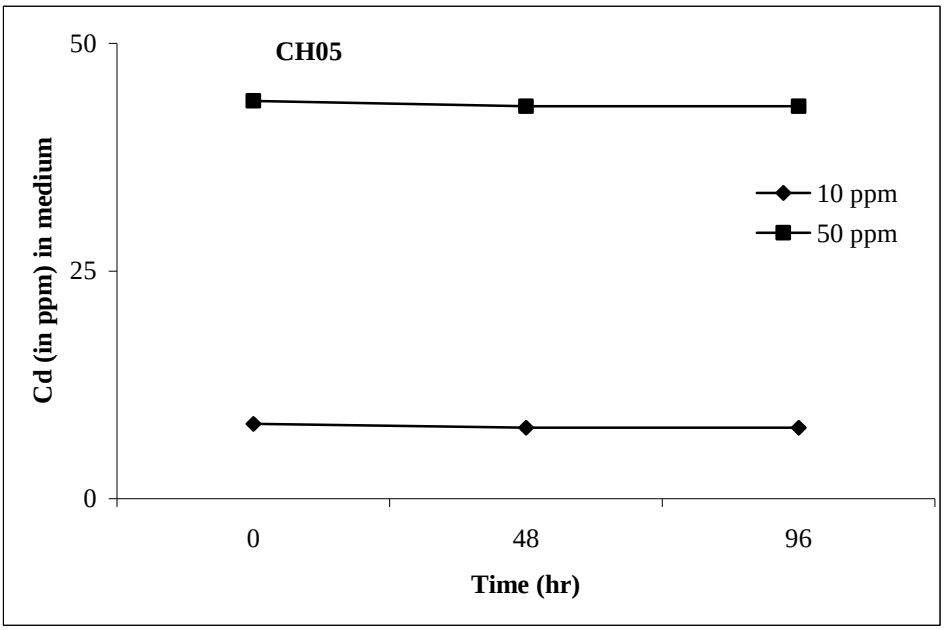
Figures 4A-C. SEM and EDS of cells of isolate GP13 grown in (A) SWNB and (B) SWNB amended with 50 ppm Pb. Panel C confirms a Pb signal measured by EDS from cells in (B).


CH07
100
75
50
25
0
Cd (ppm) in medium
0
24
48
72
96
Time (hr)

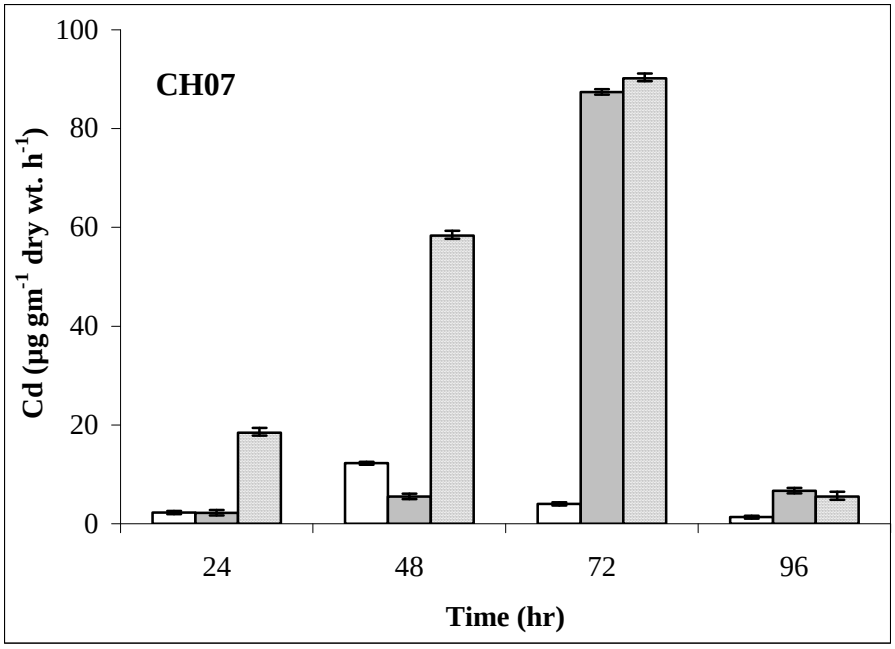
1A.


GP06
100
75
50
25
0
Cd (ppm) in medium
10 ppm
50 ppm
100 ppm
0
24
48
72
96
Time (hr)

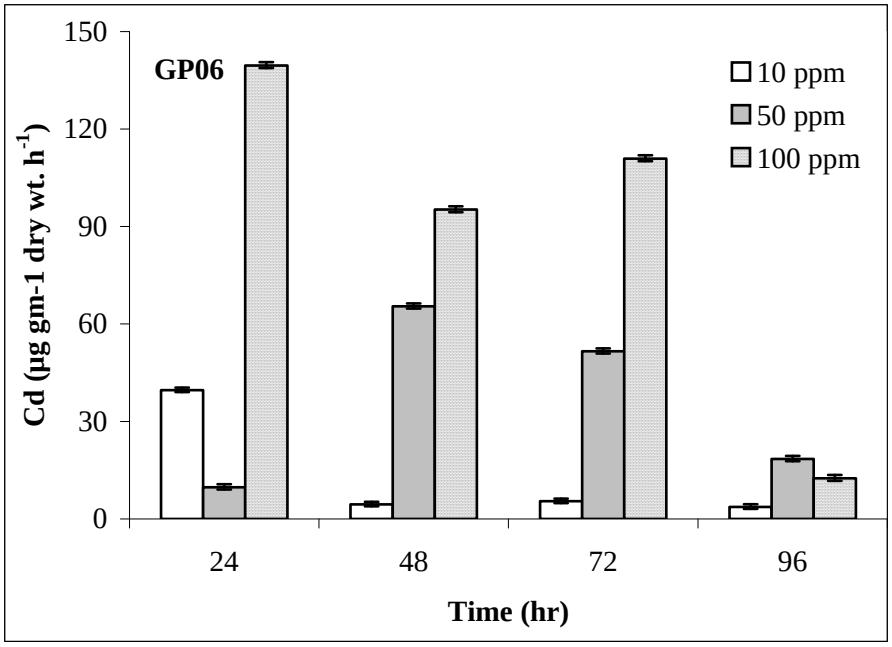
1B.



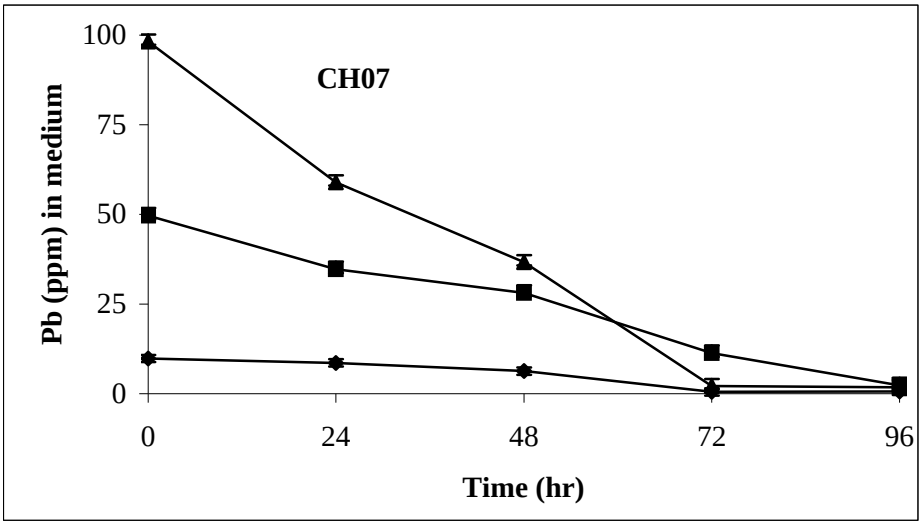
1C.


CH07
Cd (µg gm-1 dry wt. h-1)
100
80
60
40
20
0
24
48
72
96
Time (hr)

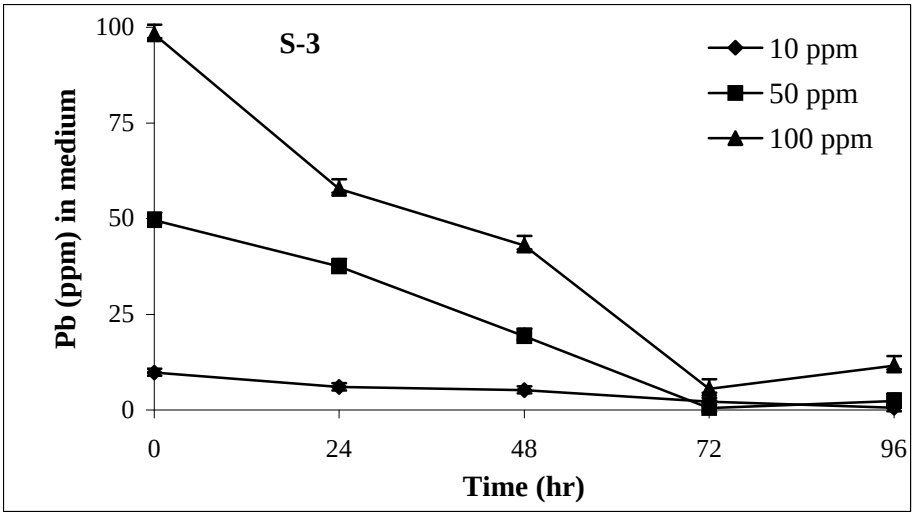
2A.


GP06
10 ppm
50 ppm
100 ppm
Cd (µg gm-1 dry wt. h-1)
150
120
90
60
30
0
24
48
72
96
Time (hr)

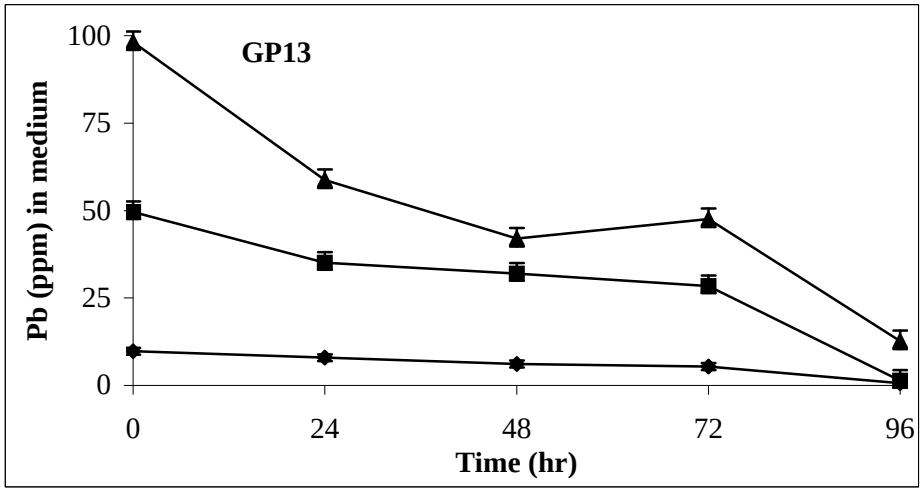
2B.


CH07
Pb (ppm) in medium
100
75
50
25
0
0
24
48
72
96
Time (hr)

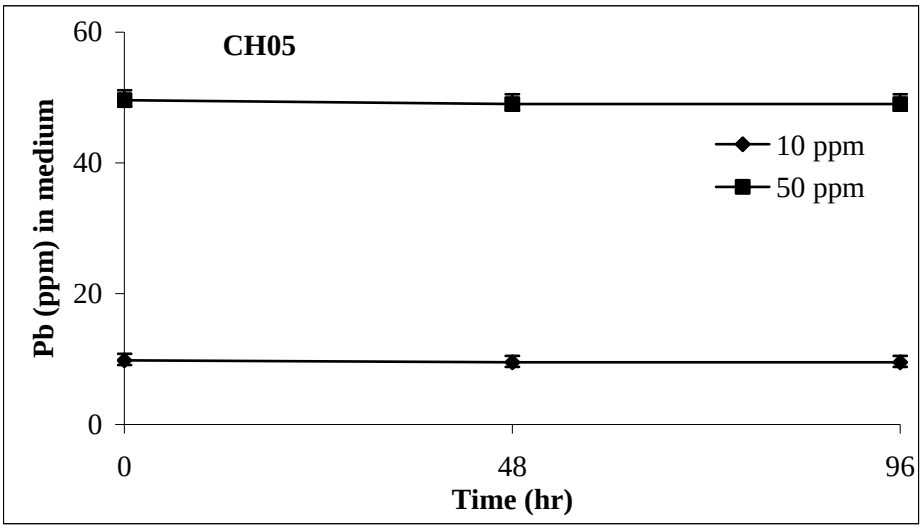
3A.


S-3
10 ppm
50 ppm
100 ppm
Pb (ppm) in medium
100
75
50
25
0
0
24
48
72
96
Time (hr)

3B.


GP13
Pb (ppm) in medium
100
75
50
25
0
0
24
48
72
96
Time (hr)

3C.


CH05
Pb (ppm) in medium
60
40
20
0
0
48
96
Time (hr)
10 ppm
50 ppm

3D.

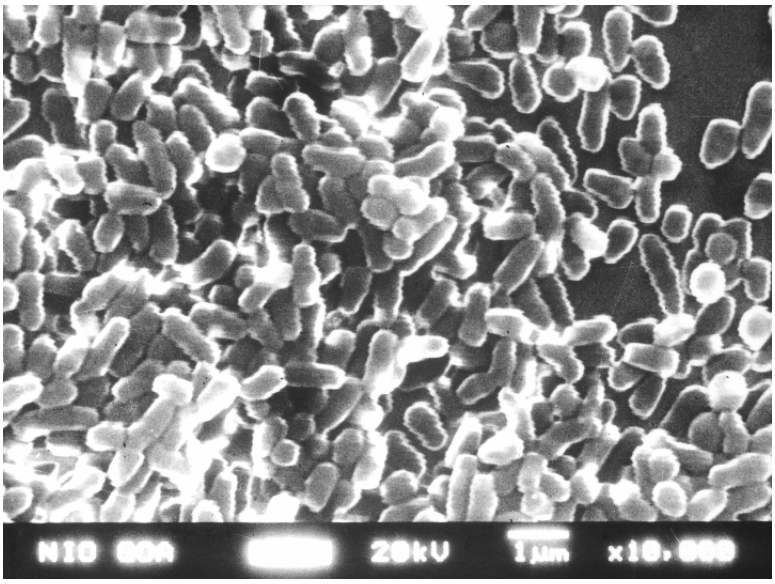


Figure 4A

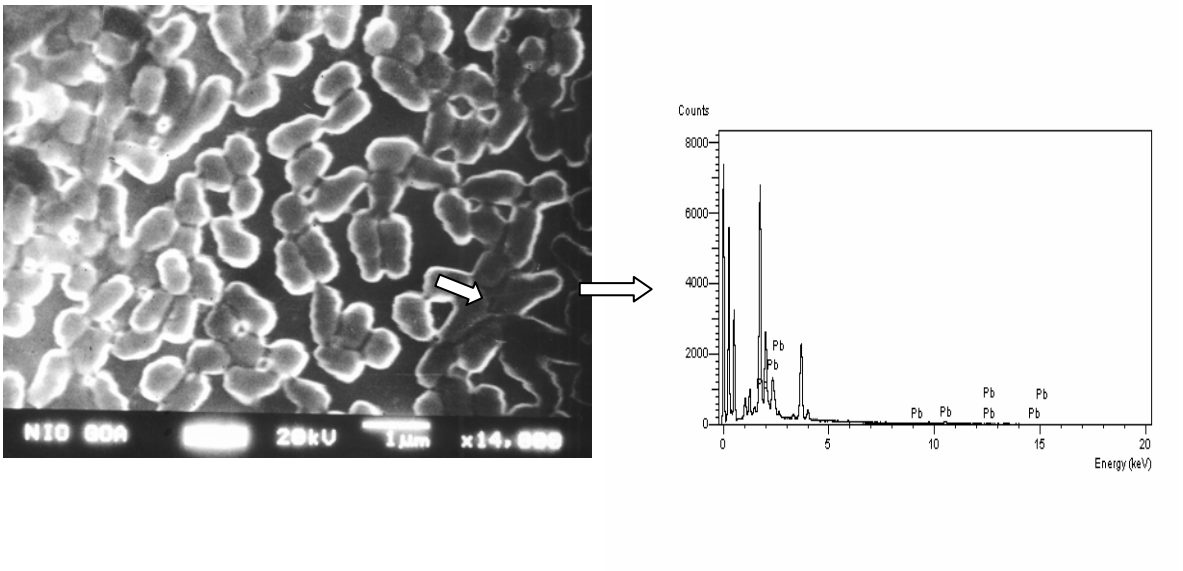


Figure 4B **Figure 4C**