In situ Protein Reactivity Profile of Intracellular Protein Targeted by Pb, Cd in Heavy-Metal-Tolerant Marine Sediment Bacteria Isolated from Iligan Bay

Mannix P. Balanay\textsuperscript{1*}, Myrna S. Mahinay\textsuperscript{1*}, Arvin B. Balala\textsuperscript{1}, Anita P. Rivera\textsuperscript{1}, and Alexes C. Daquinag\textsuperscript{3}

\textsuperscript{1}Department of Chemistry  
College of Science and Mathematics  
Mindanao State University – Iligan Institute of Technology  
Iligan City 6200, Philippines  
\textsuperscript{2}Department of Chemistry  
College of Arts and Sciences  
Silliman University  
Dumaguete City 6200, Philippines  
\textsuperscript{3}Molecular Physiology & Biophysics  
Baylor College of Medicine  
Houston, Texas USA

The current research has shown promising potential for the application of the endemic, indigenous, non-pathogenic, and heavy-metal-tolerant marine sediment bacteria isolated from Iligan Bay in the bioremediation of solid waste or wastewater contaminated with the heavy metals Pb and Cd. Optical density, wet-weight differential, paper disc diffusion, biosorption efficiency, quantification of the metallothionein, and \textit{in situ} protein reactivity profile using the 10\% SDS-PAGE have shown a potential of metal uptake by the bacteria. Reactivity with the target protein having the MW of \textasciitilde39 kDa formed the complex having a MW of \textasciitilde42 kDa.

\textbf{Keywords}: bioremediation; \textit{in situ} protein profile; metallothionein; metal-binding motif; biosorption efficiency

\section*{INTRODUCTION}

In the southern Philippines, Iligan City has been the home to a number of large industries. Its Iligan Bay is a catch-basin to a number of environmental pollutants, including heavy metals. Since the natural mineralization of metals is a slow process, pollution of heavy metals constitutes one of the most important environmental problems of industrial societies [1-3]. The mineralization and concentration of metals from toxic industrial wastes has been demonstrated in biomass adsorption. This strategy makes use of biological organisms such as the microorganisms to decontaminate the industrial waste from toxic heavy metals [2,3].

\*Authors to whom correspondence should be addressed
Several bacteria have shown to accumulate a high percentage of heavy metal species. These microorganisms are known to oxidize, reduce, biosorb, bioaccumulate, and bioprecipitate many transition metals by forming metal-binding colloids and films. They affect transition metal speciation by causing changes in pH, electrochemical potential, anion availability or by catabolizing metal chelating agents. In polluted ecosystems, bioremediation is one particular valuable approach to remove or detoxify pollutants using organisms. Perhaps, the best-known example of marine bioremediation was provided during the clean up of the Exxon Valdez oil spill, when fertilizer was used to enhance the breakdown of oil by naturally occurring bacteria [2,3].

Previous investigation by Balala [4] had found some potential toxic-metal tolerant marine sediment bacteria that are endemic and prolific in the industrial effluents of Iligan Bay. The full taxonomic identification of these endemic bacteria is yet to be performed. These bacteria are endemic and are found abundantly available at site for sampling and further studies.

In this research, the bioremediation potential of the bacteria with toxic-metals (Pb and Cd) and the intracellular protein reactivity target of the toxic metal by in situ protein profiling are investigated. The interest on protein studies for these bacteria is on the premise that in nature, many proteins, which are relatively abundant, kinetically labile, and thermodynamically stable, have stable units of metalloprotein active centers. These facilitate rapid assembly or disassembly of the protein as well as the dissociation or association of the substrates via energy-driven processes. Metal-binding cofactors have evolved to enhance the bioavailability of metal ions and entry into the cell can occur via passive diffusion or through ion-channels [5].

Target identification of protein(s) with reactivity to metal presents a great challenge in chemical genomics experiments. Without this knowledge, subsequent efforts to elucidate the mechanism of action of toxicity of metal within the cell, as well as to refine the structure-function relationship for effective bioremediation applications, are seriously impeded.

**METHODOLOGY**

The three marine sediment bacteria (*Bacillus* sp. 1, *Bacillus* sp. 2, and *Chryseomonas* sp. 2) from Iligan Bay were isolated previously [4] and were used in this study. Inasmuch as these bacteria from the previous study [4] have no full taxonomic study, the current research coded the bacteria as BBPb-1, BBPb-2 and BCCd-1 for *Bacillus* sp. 1, *Bacillus* sp. 2 and *Chryseomonas* sp. 2 respectively.

**Growth Response Behavior**

**Optical Density Method.** Optical density was determined at $\lambda=550$ nm using Spectronic 20D+ Milton Roy Spectrophotometer for 5 mL MS Broth (1 % Tryptone, 0.8 % NaCl, 0.1 % Yeast Extract, and 0.1 % D(+)-Glucose; autoclaved at 15 psi for 15 min) with the bacterial isolates. An absorbance reading of approximately 0.2 was optimized for all the MS Broth in the control and the broths prior to addition of the metal solutions. Appropriate volumes of the metal solutions (Pb(NO$_3$)$_2$ for BBPb-1 and BBPb-2 and Cd(NO$_3$)$_2$ for BCCd-1) (0 – 100 ppm) were added to the MS Broth containing the bacteria for the metal-dosed set-ups. All were incubated for 24 h at 37°C with constant shaking [6-7]. Four independent trials were performed for the control and each of the metal-dosed experiments.

**Wet-weight Differential Method.** The bacterial isolates inoculated in the MS Broth (5 mL) were incubated with shaking at 37°C and serve as the control. Simultaneously, MS Broth cultures inoculated with the bacterial isolates were added with heavy metal solution ranging from 0 to 100 mg L$^{-1}$ concentrations. Incubation at 37°C follows for 24-hours period after taking into account the weight (0.1 mg) of the tubes before incubation. The cultures were centrifuged in the refrigerated centrifuge machine (Jouan Centrifuge MR 231) at 30,000xg and the wet-pellets were weighed [6-7]. For the control and each of the metal-dosed experiments, four independent trials were performed.

**Paper Disc Diffusion Method.** A 5 mL top agar of 0.5 % Agar and 0.6 % NaCl was inoculated with the bacterial isolate. The top agar was aseptically poured to the previously prepared MS Agar plates (1 % Tryptone, 0.8 % NaCl, 0.1 % Yeast Extract, 1 % Agar and 0.1 % D(+)-Glucose;
autoclaved at 15 psi for 15 minutes). A Whatman #3 filter paper (6 mm dia.) previously impregnated with heavy metal solutions was aseptically placed at the center of the inoculated plate. Incubation of the plates followed for 24 h at 37°C. After the incubation period, each plate was monitored for the zone of growth or the zone of inhibition at the vicinity of the impregnated paper disc [8]. Quadruplicate experiments were performed.

**Biosorption Efficiency**

10 mL MS Broth containing from 0 to 100 ppm heavy metal concentrations were inoculated with the bacterial isolates, followed by incubation for 24 h at 37°C, with constant shaking. The broths were centrifuged at 30,000 x g at 4°C for 10 min. The bacterial pellet was washed twice with Phosphate Buffer solution (pH 7). Repeated centrifugation was performed and then decanted to separate the supernatant. The cell pellets were discarded and the supernatant liquid was acid digested (conc. HNO₃ and dilute 6N HCl) [9]. Analysis of the heavy metals were done by Anodic Stripping Voltammetry [10-11] using Metrohm™ 693 VA Processor with Metrohm™ 694 VA Stand. Four independent trials were performed for the control and each of the metal-dosed experiments.

The amount of heavy metal uptake by the bacteria was determined from the metal concentration that remained in the supernatant broth. Biosorption Efficiency was calculated using the formula [12]:

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\text{Biosorption Efficiency} = \frac{C_i - C_f}{C_i} \times 100
\]

where \(C_i\) was the amount of heavy metal initially present in the supernatant of the culture broth before bacterial incubation; \(C_f\) was the amount of heavy metal left in the supernatant after 24 h of bacterial incubation minus the amount of metal present in the culture broth alone.

**Electrochemical Detection of Metallothionein**

MS Broth (5 mL) were inoculated with each of the bacterial isolates and incubated for 24 h, with shaking, at 37°C. Simultaneously, MS Broth cultures with the different bacterial isolates were added with optimum heavy metal concentration (10 ppm Pb for BBPb-1, 20 ppm for BBPb-2, and 10 ppm Cd BCCd-1) and were incubated for 24 h, with shaking, at 37°C. The isolates were centrifuged at 30,000 x g at 4°C for 10 min and the supernatant was discarded. The cell was subjected to freeze drying (using Yamato Freeze Dryer DC41) to remove the remaining broth. 200 mg of freeze dried cells of the bacterial strains were re-suspended with 1 mL of deionized water. 1 mL of Laemmli sample treatment buffer (2X, 0.125 M Tris-Cl, 4 % SDS, 20 % v/v glycerol, 0.2 M Dithiothreitol (DTT), 0.02 % bromophenol blue, pH 6.8) was then added, and the samples were boiled at 95°C for 4 min.

Cellular debris and insoluble materials were removed by centrifugation at 30,000 x g at 4°C for 10 min. The supernatant was transferred and quantitative determination of the metallothionein was performed by Differential Pulse Polarography. Polarographic determination [13-15] of MT was performed using Metrohm 693 VA Processor with 694 VA Stand connected to a static mercury drop electrode, using a modification of the Brdicka procedure described by Thomposon and Cosson [14]. Each sample was purged with nitrogen for 30 s before analysis to mix sample and electrolyte. The scanned potential range was -1.4 to -1.70 V at 5 mV/s and the modulation amplitude was 50 mV. The drop time for the mercury electrode was set to 1 s. A standard curve was prepared with rabbit MT-1 (Sigma Chemical Co.) from rabbit liver as standard [15].

**In situ Protein Reactivity Profile**

Whole-cell total proteins were extracted from 10 mL 24-h cultures (MS Broth - 1% Tryptone, 0.8 % NaCl, 0.1 % Yeast Extract, and 0.1 % D(+)-Glucose; autoclaved at 15 psi for 15 min) was inoculated with the marine sediment bacteria from MS Agar plates (1 % Tryptone, 0.8 % NaCl, 0.1 % Yeast Extract, 1 % Agar and 0.1 % D(+)-Glucose; autoclaved at 15 psi for 15 minutes) incubated at 37°C of bacterial isolates with and without heavy metals. The cells were centrifuged for 10 min at 30,000 x g at 4°C in a Jouan Centrifuge MR 231. The bacterial pellet was washed twice with PBS and was centrifuged.

The pellet was re-suspended in 100 μL deionized water. One hundred microliter of the Laemmli sample treatment buffer (2X, 0.125 M Tris-Cl, 4 % SDS, 20 % v/v glycerol, 0.2 M Dithiothreitol (DTT), 0.02 % bromophenol blue, pH 6.8) was
then added, and the samples were boiled for 4 min. Cellular debris and insoluble materials were removed by centrifugation. The protein extracts from the supernatant liquid were measured for protein content using UV-VIS Spectrophotometer Shimadzu UV-160A at a wavelength of 280 nm. Bovine Serum Albumin (BSA) was used as protein standard to determine the concentration of the protein of the bacterial lysates [16].

Protein samples were run using the 10% SDS-Polyacrylamide Gel Electrophoresis (Linear Slab Gel) method in an Atto AE-6400 vertical unit with 1 mm gel spacers. Cell lysates containing 20 µg protein were loaded into each well. The Coomassie Brilliant Blue standard staining method was used for visualization. A Mark 12™ (Invitrogen Corp) MW protein standard or Broad Range protein standard (Bio-Rad Laboratories) was used as molecular weight markers [16].

RESULTS AND DISCUSSION

Balala isolated some marine sediment bacteria from Iligan Bay that exhibited tolerance to toxic metals [4]. Three of these bacteria (Bacillus sp. 1 and 2 and Chryseomonas sp. 2) were found to exhibit higher tolerance to Pb and Cd.

Studies on the bacterial growth response behavior of these three marine sediment bacteria were first approached by observation of the optical density of the broth culture that was dosed with different concentrations (0-100 ppm) of the toxic metals, Pb and Cd. The quantitative plots of the data for the three bacterial isolates (Figure 1) clearly show optimum bacterial growth responses at 10 ppm Pb, 20 ppm Pb and 10 ppm Cd for BBPb-1, BBPb-2 and for BCCd-1, respectively.

The same optimum metal-concentration levels were confirmed in the wet-weight differential method and the biosorption efficiency. A higher absorbance was observed at 0.367 and a large weight difference of the harvested bacterial pellets (0.217 grams) at the optimum concentration of 10 ppm Pb relative to the other concentrations for BBPb-1. Similar results were observed for BBPb-2 having an optical density of 0.250 and 0.163 grams weight differential at 20 ppm Pb. The BCCd-1 also exhibited an absorbance of 0.068 and 0.152 grams weight difference at 10 ppm Cd. These results suggest a favorable growth of the bacteria at the optimum concentration, hence, the increase in the cell density in the broth culture as shown by the absorbance and in the weight of the harvested cell pellets.

Fig. 1. Heavy metal-dose concentration (ppm) plots against (A) change in optical density (absorbance), (B) wet-weight difference (grams), (C) the % bio-sorption efficiency of the bacterial isolates BBPb-1, BBPb-2 and BCCd-1.

The biosorption efficiency results also confirm the same observations at the optimum concentration where 90.46 ±1.69%, 77.01 ± 2.40% and 63.62 ± 0.09% were the percent biosorption efficiency.
obtained for BBPb-1, BCCd-1 and BBPb-2, respectively. The results suggest that the heavy metal uptake by the three bacteria is greater at the optimum concentration, which was confirmed by the differential pulse polarography electrochemical detection of the expressed metallothionein. The biosorption efficiency experiments focus on the metals remaining in the supernatant solution. The quantification of the expressed metallothionein correlates to the intracellular uptake of the metal but not to the metal bound at the cell membranes. Hence, the amount of metals adsorbed by the bacterial cell walls or covalently bound to the receptor sites of the bacterial cell walls were not quantified in this study. These were deposited as cellular debris in the centrifugates, after breaking the cell walls to extract the proteins present in the cytosol. The optimum metal concentration responses for these three marine sediment bacteria confirm the tolerance of these bacteria with Pb and Cd in the previous study by Balala [4]. Figure 1 clearly illustrates the trends, i.e., they consistently decline after the optimum concentration has been reached. This suggests toxicity of the bacterial isolates as the concentration of the metal is increased beyond the optimum concentration. This also indicates the ineffectiveness of the bacterial isolates in the removal of the toxic metal, when its concentration is higher than the optimum concentration, as this would lead to the eventual death of the bacteria and, therefore, limited the tolerance of the bacteria to the toxic metal at the optimum concentration [1-3,22-23].

The paper disc diffusion method showed the zone of bacterial growth at the vicinity of the paper disc impregnated with the metal up to the optimum concentration, while a zone of bacterial inhibition was observed at the metal concentrations beyond the optimum. Figure 2 shows representative results of the paper disc diffusion response behavior to the toxic metal treatment of the isolates, exhibiting a zone of growth promotion surrounding a filter paper disc impregnated with the heavy metal. The growth around the paper disc suggests affinity of the bacteria at the vicinity of the paper disc impregnated with the metal up to the optimum concentration. The zone of growth promotion suggests that the heavy-metal tolerant bacteria would best survive or grow in an environment with appropriate concentration of metals and be able to utilize the heavy metals.

Metallothionein (MT) is expressed in large amounts when excess quantities of certain metal ions including toxic ones such as Cd\(^{2+}\) or Pb\(^{2+}\), are present in the cells [5]. It is found to act as a
detoxifying agent, where its biosynthesis is part of a tightly regulated system for controlling the levels of metal ions in vivo when metal ions induce the production of metallothionein by activation of specific genes [27]. It has long been recognized that there is a reduction in the toxicity of metal ions following the induction of MT in animals. Comparative tolerance to metals of cultured cells with elevated levels of MT has also been described frequently [5,13,27].

Differential pulse polarography (DPP) has been used to analyze MT in biological samples. The method is based on the detection of the changes in the current that occurs when a compound is either oxidized or reduced. The reduction of the sulfhydryl group of MT is measured in DPP. Since sulfhydryl-containing proteins other than MT will contribute to the signal, the specificity of DPP is highly dependent upon sample preparation. High molecular-weight proteins are removed from the samples by precipitating it out by heat denaturation, leaving the heat stable MT in solution. Also the method is not sensitive to small sulfhydryl-containing compounds such as glutathione. The major advantage of DPP is that the analysis is not influenced by the metal composition of the sample. Purified MT from crabs or rabbits has been used as standards for the reduction of the sulfhydryl group of MT [13-15].

The electrochemical detection of the reduced metallothionein by DPP would yield a direct relationship with the amount of the metallothionein (MT) expressed inside the cell upon intracellular metal uptake by the bacteria [13-15]. In other words, the amount of metallothionein expressed in the cell increases with increasing intracellular uptake of the toxic metal. The amount of MT obtained for the three bacterial isolates directly correlate with the results obtained in the biosorption efficiency study as that shown in Figure 1.

The BBPb-1 isolate has 90.46 ± 1.69 % biosorption efficiency, followed by BCCd-1 which has 77.01 ± 2.40% and BBPb-2 at 63.62 ± 0.09%. These results (shown in Figure 3) correlate with the amount of MT expressed by BBPb-1 showing the highest differential in the MT concentration (concentration difference of 906.8 μg/g MT) of the metal-dosed bacteria than the control with 26.6% difference. The BCCd-1 has the concentration difference of 315.0 μg/g MT which is 10.6% differential, while the least differential is seen in BBPb-2 with 7.2 % (concentration difference of 201.4 μg/g MT).

The quantification of MT in the bacterial lysates of the control and the metal-dosed bacteria show that BBPb-1 bacteria produce more metallothionein in response to higher metal uptake (having the larger difference), while BBPb-2 with the least biosorption efficiency has shown the lower difference in the metallothionein content. Thus, of the two Bacillus sp. 1 and 2 isolates, the BBPb-1 has the highest metal biosorptive potential for Pb, while the Chryseomonas sp. 2 coded as BCCd-1 has metal biosorptive potential for Cd. The observed optimum concentration of 10 ppm and 20 ppm is way beyond the threshold levels set by the Department of Environment and Natural Resources (DENR) for the surface water (class: SA, SB, SC) quality standard of lead and cadmium at 0.05 ppm and 0.01 ppm respectively [28].

A significant implication is that for these endemic bacteria, their high biosorptive capacity of the toxic metal represents a great potential in the removal of toxic metals from the surface water up to its optimum concentration. The results also support the literature reports on the intracellular level of the metal in the organism being the rate-limiting factor in the growth kinetics of the bacteria and not the metal concentration in the surrounding water [16,23-26].

Having established the optimum concentrations of toxic metal tolerance of the three bacterial strains and being confirmed, an in situ protein reactivity profiling was performed to establish the identity of the target protein(s) that could have interacted with the metal upon its intracellular uptake. The in situ protein reactivity profile is performed to identify proteins uniquely labeled by metal ions or bioactive compounds in natural products or the synthetic analogs of compounds that can only occur in the context of the living cells.
The 10% SDS-PAGE in situ protein reactivity profiles of the toxic metal (Pb and Cd) with the three marine sediment bacteria after uptake are shown in Figures 4, 5, and 6. Figure 4 shows the 10% SDS-PAGE in situ protein reactivity profile of BBPb-1 bacterial lysates showing the control (0 ppm Pb) coded as BBPb1-0 and bacterial lysates dosed with 10 ppm Pb (coded as BBPb1-10). The result showed distinct band of the protein in the control BBPb1-0 (0 ppm) and that of the metal-dosed BBPb1-10 (10 ppm Pb) that shifted to higher MW region. Most of the proteins found in the profile of the control were not altered or shifted and has remain constant in its location as shown in the SDS-PAGE of the metal-dosed. Compared to the control, the SDS-PAGE gel of the metal-dosed sample showed the appearance of the protein band at ~42 kDa and the disappearance of the ~39 kDa protein band. This SDS-PAGE confirms that there was an intracellular biosorption of the heavy-metal Pb by the bacterial strain BBPb-1, which interacted with the proteins to form the metal-protein complex having the molecular mass (MW) of ~42 kDa.

The same results were obtained with BBPb-2 bacterial lysates when dosed with Pb 20 ppm Pb as shown in Figure 5. The SDS-PAGE of the metal-dosed also indicated the appearance of the protein band at ~42 kDa and the disappearance of the ~39 kDa protein band. Again, the results indicated Pb metal uptake in the cell that reacted with the protein at ~39 kDa to form the metal-protein complex with the appearance of the band at ~42 kDa.
Figure 6 also showed the SDS-PAGE results for the BCCd-1 when dosed with cadmium at the optimum concentration. Similar results were obtained where the protein band at ~42 kDa is formed and the fading out of the ~39 kDa protein band.

In this study, the three marine sediment bacteria were found to have similar reactivity of the metal to the intracellular protein where both Pb and Cd metals have reactivity to the protein having the molecular mass of ~39 kDa to form the metal-protein complex with the appearance of the ~42 kDa band.

CONCLUSIONS AND RECOMMENDATIONS

The research has shown promising potential for the application of the endemic and indigenous non-pathogenic marine sediment bacteria in the implementation of the bioremediation of solid waste or wastewater contaminated with the heavy metal, especially Pb and Cd. The observed optimum biosorption capability of the endemic bacterial strain has the potential to detoxify toxic metal in the surface water up to the threshold level set as safe by the DENR.

The formation of the ~42 kDa band is indicative of the metal-protein complex formed by the interaction of the ~39 kDa protein with the metal Pb and Cd. However, the structure of such complex is yet to be determined or elucidated in another research. It is recommended that the protein (MW ~39 kDa) and the metal-protein complex having the molecular mass of ~42 kDa be isolated, followed by in situ protein reactivity profile with the metals.

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